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THE γ-TUBULIN GENE FAMILY IN HOMO SAPIENS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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*****

The Ohio State University
1999

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ABSTRACT

γ Tubulin is an essential component of the microtubule organizing centers of eukaryotes. It is required for the nucleation of microtubule assembly from microtubule organizing centers in animal and fungal cells, and it is, consequently, essential for mitosis and for the organization of the microtubule cytoskeleton in these organisms. Some of the most useful cancer chemotherapy agents target microtubules, and it has been proposed that γ-tubulin may be the site of action for some experimental cancer chemotherapy agents (Bai et al., 1993). In spite of the importance of γ tubulin, little is known of the number and expression patterns of γ-tubulin genes in humans.

Zheng et al. (1991b) identified a human γ-tubulin cDNA (HY1) from a HeLa library. To determine if other γ-tubulin genes are present in the human genome, I have carried out Southern hybridization analysis of HeLa DNA. The results indicate that there are probably no more than three γ-tubulin sequences in the human genome. I have screened a HeLa cDNA library and isolated cDNA clones that contain partial sequences of a novel γ-tubulin gene that I have named HY2. In order to obtain a full-length copy of HY2, I have screened the human expressed sequence tag (EST) database (dbEST) and have obtained a clone I have designated HY2EST. I have also identified a processed pseudogene
of Hγ1. This is the first γ-tubulin pseudogene identified in any organism.

Hγ1 has been mapped to chromosome 17 by several labs involved in the search for the BRCA1 breast cancer susceptibility gene (Rommens et al., 1995; Friedman et al., 1995). I present data localizing Hγ2 to the same 700 Kb Yeast Artificial Chromosome that carries Hγ1.

Finally, I have analyzed the expression patterns of Hγ1 and Hγ2 in several human tissues by reverse transcriptase-polymerase chain reaction analyses. Both Hγ1 and Hγ2 are expressed in all tissues examined.
Dedicated to my mother, Gwendolyn O’Neal, who loved me first and loves me most; my grandparents, Laura and Isaiah Sneed, who showed me how to love unconditionally; my sister, Elisha O’Neal, who loves me in spite of myself; my dad, Carlton O’Neal, who learned to love me; and to my husband, Raymond Wise, who chose to love me and taught me the meaning of true love.
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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>SECTION</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>VITA</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiv</td>
</tr>
<tr>
<td>CHAPTERS:</td>
<td></td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Microtubules</td>
<td>1</td>
</tr>
<tr>
<td>General structure and function</td>
<td>1</td>
</tr>
<tr>
<td>Microtubule dynamics</td>
<td>6</td>
</tr>
<tr>
<td>Tubulin</td>
<td>11</td>
</tr>
<tr>
<td>General characteristics of tubulin</td>
<td>11</td>
</tr>
<tr>
<td>Multigene families of α and β tubulins</td>
<td>12</td>
</tr>
<tr>
<td>Functional significance of multiple tubulin isotypes</td>
<td>15</td>
</tr>
<tr>
<td>Microtubule organizing centers (MTOCs)</td>
<td>18</td>
</tr>
<tr>
<td>Topic</td>
<td>Page</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Centrosomes</td>
<td>19</td>
</tr>
<tr>
<td>Spindle pole bodies</td>
<td>24</td>
</tr>
<tr>
<td>Plant MTOCs</td>
<td>26</td>
</tr>
<tr>
<td>(\gamma) Tubulin</td>
<td>27</td>
</tr>
<tr>
<td>Discovery of (\gamma) tubulin</td>
<td>27</td>
</tr>
<tr>
<td>Ubiquity</td>
<td>28</td>
</tr>
<tr>
<td>Cellular localization and function</td>
<td>29</td>
</tr>
<tr>
<td>Biochemical characterization of (\gamma) tubulin and the (\gamma)TuRC</td>
<td>33</td>
</tr>
<tr>
<td>Multigene families of (\gamma) tubulins</td>
<td>39</td>
</tr>
<tr>
<td>Rationale for my dissertation research</td>
<td>41</td>
</tr>
<tr>
<td><strong>2. IDENTIFICATION, CLONING AND ANALYSIS OF ADDITIONAL (\gamma)-TUBULIN SEQUENCES IN <em>HOMO SAPIENS</em></strong></td>
<td>43</td>
</tr>
<tr>
<td>Introduction</td>
<td>43</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>49</td>
</tr>
<tr>
<td>Isolation of HeLa genomic DNA</td>
<td>49</td>
</tr>
<tr>
<td>Southern analysis</td>
<td>49</td>
</tr>
<tr>
<td>Sequence analysis of a genomic human (\gamma)-tubulin clone (H(\gamma)F)</td>
<td>51</td>
</tr>
<tr>
<td>Library screening</td>
<td>51</td>
</tr>
<tr>
<td>EST database search, subcloning and sequencing of a full-length (H\gamma2) clone</td>
<td>52</td>
</tr>
<tr>
<td>Construction of plasmid pASH(\gamma)2</td>
<td>53</td>
</tr>
<tr>
<td>Chromosomal mapping</td>
<td>55</td>
</tr>
<tr>
<td>Preparation of YAC DNA for PCR analysis</td>
<td>57</td>
</tr>
<tr>
<td>PCR analysis and DNA sequencing of H(\gamma)F</td>
<td>59</td>
</tr>
<tr>
<td>Blast search for H(\gamma)F</td>
<td>60</td>
</tr>
<tr>
<td>Results</td>
<td>61</td>
</tr>
<tr>
<td>Estimation of the number of (\gamma)-tubulin sequences in <em>Homo sapiens</em></td>
<td>61</td>
</tr>
<tr>
<td>Identification and characterization of a novel human (\gamma)-tubulin cDNA, (H\gamma2)</td>
<td>62</td>
</tr>
<tr>
<td>Somatic cell and radiation hybrid mapping of (H\gamma2)</td>
<td>80</td>
</tr>
<tr>
<td>(H\gamma F) is a fragment from a (\gamma)-tubulin pseudogene</td>
<td>92</td>
</tr>
<tr>
<td>Discussion</td>
<td>104</td>
</tr>
<tr>
<td><strong>3. ANALYSIS OF THE EXPRESSION PATTERNS OF (\gamma)-TUBULIN GENES IN <em>HOMO SAPIENS</em></strong></td>
<td>107</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Introduction</td>
<td>107</td>
</tr>
<tr>
<td>Materials and methods</td>
<td>109</td>
</tr>
<tr>
<td>Strains and media</td>
<td>109</td>
</tr>
<tr>
<td>Plasmid pASHγ2F construction</td>
<td>109</td>
</tr>
<tr>
<td>S. pombe transformation</td>
<td>111</td>
</tr>
<tr>
<td>Isolation of total RNA from S. pombe strains</td>
<td>112</td>
</tr>
<tr>
<td>Isolation of total RNA from HeLa cells and human tissues</td>
<td>112</td>
</tr>
<tr>
<td>RT-PCR analysis</td>
<td>113</td>
</tr>
<tr>
<td>Results</td>
<td>114</td>
</tr>
<tr>
<td>Rationale for primer design and controls for RT-PCR</td>
<td>114</td>
</tr>
<tr>
<td>Expression patterns of γ-tubulin genes in human tissues</td>
<td>120</td>
</tr>
<tr>
<td>Discussion</td>
<td>123</td>
</tr>
<tr>
<td>Bibliography</td>
<td>127</td>
</tr>
</tbody>
</table>


**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Organisms from which γ-tubulin genes or cDNAs have been isolated</td>
<td>30</td>
</tr>
<tr>
<td>2. Components of γTuRCs in different organisms</td>
<td>38</td>
</tr>
<tr>
<td>3. Human α- and β-tubulin gene chart</td>
<td>45</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Microtubule lattice arrangements</td>
<td>5</td>
</tr>
<tr>
<td>2.</td>
<td>Low stringency Southern analysis of human genomic DNA using a 5'-Hy1 probe</td>
<td>64</td>
</tr>
<tr>
<td>3.</td>
<td>Low stringency Southern analysis of human genomic DNA using a 3'-Hy1 probe</td>
<td>66</td>
</tr>
<tr>
<td>4.</td>
<td>The nucleotide sequence of HyF and its amino acid sequence</td>
<td>68</td>
</tr>
<tr>
<td>5.</td>
<td>A comparison of the predicted amino acid sequences of HyF and Hy1</td>
<td>71</td>
</tr>
<tr>
<td>6.</td>
<td>The nucleotide and predicted amino acid sequence of clones pDOW7 and pDOW21</td>
<td>73</td>
</tr>
<tr>
<td>7.</td>
<td>The nucleotide and predicted amino acid sequence of clones pDOW1a and pDOW12</td>
<td>76</td>
</tr>
<tr>
<td>8.</td>
<td>A comparison of the predicted amino acid sequences of Hy2-clone pDOW7 and Hy1</td>
<td>78</td>
</tr>
<tr>
<td>9.</td>
<td>The nucleotide sequence of Hy2EST and its predicted amino acid sequence</td>
<td>82</td>
</tr>
<tr>
<td>10.</td>
<td>The construction of plasmid pASHγ2</td>
<td>85</td>
</tr>
<tr>
<td>11.</td>
<td>A comparison of the predicted amino acid sequences of Hy2EST</td>
<td>xiv</td>
</tr>
</tbody>
</table>
12. A comparison of the nucleotide sequences in the 3'UTR regions of Hy1 and Hy2

13. Schematic diagram of human chromosome 17 showing Hy1, BRCA1, and Hy2

14. PCR analysis of HSD2 YAC DNA with Hy2 specific primers

15. A comparison of the predicted amino acid sequences of HyF and Hy1 with the HyF specific primers underlined

16. PCR amplification of HyF from HeLa cell and human genomic DNA using HyF specific primers

17. A comparison of the nucleotide sequence of human genomic clone "DJ1165K" with Hy1 and HyF

18. A comparison of the nucleotide sequences found in the 3'UTRs of Hy1 and Hy2

19. The construction of plasmid pASHyF and S. pombe strain HMHG2

20. PCR analysis of total RNA isolated from three S. pombe strains used as controls for RT-PCR

21. RT-PCR analysis of the expression patterns of Hy1 and Hy2 in several human tissues
CHAPTER 1

INTRODUCTION

Microtubules

General structure and function of microtubules

The eukaryotic cytoskeletal network is composed of three types of protein filaments— microtubules, actin filaments, and intermediate filaments— that provide the cell with strength, shape and the ability to coordinate movement. Microtubules are cylindrical, dynamic cellular organelles. They are involved in a variety of cellular functions including the maintenance of cellular morphology and polarity, chromosome movement during mitosis and meiosis, intracellular transport of cytoplasmic organelles and vesicles, and ciliary and flagellar motility. In cilia, flagella, and most sperm tails, microtubules are arranged in a 9+2 structure in which outer doublet microtubules surround two central pair microtubules. Centrioles and basal bodies are composed of nine microtubule triplets surrounding an amorphous core.

Microtubules vary in length and are approximately 25 nm in diameter. They are composed of two globular subunits called α and β tubulin that form a heterodimer (Bryan
and Wilson, 1971). Both monomers are approximately 50 kd in size, 450 amino acids in length and they share 36-42% amino acid identity (reviewed in Little and Seehaus, 1988). Tubulin heterodimers are arranged in a head-to-tail fashion forming longitudinal protofilaments which have a main 4 nm periodicity corresponding to the monomers and an 8 nm periodicity corresponding to the heterodimers (Amos and Klug, 1974). Adjacent protofilaments are staggered by 0.9 nm forming a 3-start left-handed helix (Amos and Klug, 1974; Linck and Amos, 1974; Mandelkow et al., 1986).

The actual nature of the lateral interactions between monomers of adjacent protofilaments was a long-standing controversy in microtubule research. Originally, Amos and Klug (1974) analyzed diffraction patterns of electron micrographs of flagella and suggested that the “A” microtubules of flagellar doublets formed what they called the “A” lattice and B-tubules formed a "B" lattice (see Figure 1a and 1b). In the A-lattice (Figure 1a), α-tubulin monomers of one protofilament and β-tubulin monomers of adjacent protofilaments interact. The A-lattice was favored as the general structure of microtubules for many years because 13-protofilament microtubules are helically symmetrical in this type of lattice. Song and Mandelkow (1993, 1995), however, used kinesin-decorated microtubules to show that the A and B tubules of flagellar outer doublets and in vitro assembled brain tubulin form a B-lattice. In the B-lattice (see Figure 1b), α-tubulin monomers of one protofilament interact with α monomers of adjacent protofilaments and β-tubulin monomers of adjacent protofilaments interact. These findings have been corroborated in vivo by Kikkawa et al. (1994) and by the high resolution model of the
microtubule proposed by Nogales et al. (1999).

In the majority of eukaryotes, microtubules are composed of 13 protofilaments (Ledbetter and Porter, 1964; Porter, 1966; Tilney et al., 1973). Exceptions to this rule have been observed in specific cell types of crayfish, Caenorhabditis elegans, guinea pigs, Drosophila melanogaster and Homo sapiens (Burton et al., 1975; Savage et al., 1989; Raff et al., 1997; Kikuchi et al., 1991). Protofilament number varies greatly when microtubules are assembled in vitro, ranging from 11 to 16 protofilament microtubules, depending upon the assembly conditions used in the experiments (McEwen and Edelstein, 1977; Scheele et al., 1982; Evans et al., 1985; Wade et al., 1990). Evans et al. (1985) demonstrated that the protofilament number appears to be established by microtubule organizing centers (MTOCs) in vitro. Interestingly, genetic studies conducted in C. elegans and D. melanogaster suggest that protofilament number may not only be established by MTOCs but can also be controlled by specific β-tubulin isotypes (Savage et al., 1989; Raff et al., 1997).

Microtubules are intrinsically polar, dynamic polymers. The plus end of the microtubule, which, in cells, is distal to the MTOC, is fast growing, while the minus end of the microtubule, which is proximal to the MTOC, is slow growing (Bergen & Borisy, 1980). The polarity of microtubules is important because it facilitates the directional movement of cargos by motor proteins. For example, conventional kinesins are plus end-directed motors, while dyneins are minus end-directed motors. These motors transport essential cargo (e.g. organelles, other proteins, chromosomes) along microtubules in a
In the A-lattice, $\alpha$-tubulin monomers of one protofilament interact with $\beta$-tubulin monomers of adjacent protofilaments. A 13-protofilament microtubule with an A-lattice will form a helically symmetrical lattice. In the B-lattice $\alpha$-tubulin monomers contact $\alpha$-tubulin monomers in adjacent protofilaments and $\beta$-tubulin monomers contact $\beta$-tubulin monomers. The lateral interactions between protofilaments causes a 13-protofilament microtubule to have a seam, where $\alpha$ tubulin and $\beta$ tubulin are in lateral contact, because one turn of a 3-start left-handed helix creates a rise of three tubulin monomers.
Figure 1.

a. A-Lattice

b. B-Lattice

Courtesy of Tetsuya Horio
directional manner (reviewed in Hirokawa et al., 1998; Barton and Goldstein, 1996).

Previous biochemical and sequence analysis of tubulin (Weisenberg et al., 1968; Geahlan and Haley, 1977) revealed that tubulin is a GTP-binding protein. The GTP on β tubulin is hydrolyzable, while the GTP on α tubulin is nonhydrolyzable (MacNeal and Purich, 1978; Spiegelman et al., 1977). More recently, Mitchison (1993) found that GTP-coated fluorescent beads bind specifically to the plus ends of microtubules indicating that the exchangeable site must be exposed at the plus end. Nogales et al. (1998a) used electron crystallography to show that the GTP binding site on α tubulin is located at the intradimer interface between the tubulin subunits, while the hydrolyzable site on β tubulin is partially exposed in the dimer but buried after hydrolysis and addition of another dimer during elongation of the microtubule (Nogales et al., 1998a, 1999). Therefore, the orientation of the tubulin dimer relative to the polarity of the microtubule lattice has been established with the β-tubulin subunit being terminal at the plus end and the α-tubulin subunit being terminal at the minus end (Nogales et al., 1999).

Microtubule dynamics

Early in this century E.B.Wilson (1925), along with other cytologists, realized that the linear fibers that make up the mitotic spindle change rapidly with time. Later Inoué and colleagues (1951) used a sensitive polarized light microscope to analyze the dynamic formation and disappearance of these mitotic fibers. They demonstrated that the protein subunits in the spindle polymer were in dynamic equilibrium with the subunits in the cell’s cytoplasm. It then became clear that a better understanding of the mechanism of
microtubule polymerization needed to be determined. Weisenberg (1972) demonstrated that microtubules can be assembled in vitro from rat brain homogenates by warming the homogenates in the presence of GTP, Mg\(^+\), and EGTA, a calcium chelator. Subsequently, Gaskin et al. (1974) used turbidity measurements to study microtubule assembly in vitro and found that assembly only occurs above a specific concentration, called the critical concentration for assembly.

Over the past twenty-five years three main theories have been advanced to explain microtubule dynamics (reviewed in Desai and Mitchison, 1997). Initially the steady-state theory of subunit polymerization postulated that when microtubules are in equilibrium with the tubulin in the cytoplasm there is constant association and dissociation of tubulin dimers at microtubule ends which allows the length of the microtubule to remain constant (Oosawa, 1962). While studying the dynamic exchange of subunits on microtubule ends, Margolis and Wilson (1978) proposed the theory of treadmilling in which a net gain of tubulin at the plus end of the microtubule and a net loss of tubulin at the minus end of the microtubule keeps the microtubule length constant at equilibrium.

Subsequently, Mitchison and Kirschner (1984a, b), inferred from length measurements of microtubules in vitro, that within a population of microtubules some were growing, while others were shrinking-- a phenomenon they called “dynamic instability”. Later, Horio and Hotani (1986) demonstrated directly, by observing individual microtubules in vitro using dark field microscopy and real-time video recording, that growing and shrinking microtubules do coexist within a population, with the individual
microtubules alternating between these two phases. Later, Walker et al. (1988) coined the term "catastrophe" to refer to the microtubules' transition from elongation to rapid shortening; whereas the reverse transition, from shortening to elongation, was called "rescue". Dynamic instability of microtubules also occurs in vivo as has been shown by Salmon et al. (1984), Saxton et al. (1984), Koshland et al. (1988) and Cassimeris et al. (1988). When compared to the dynamic instability of pure tubulin assembled in vitro, microtubules assembled in vivo polymerize at a rate that is five to ten times faster than that of pure tubulin (at a similar concentration), and they exhibit a high frequency of catastrophe (reviewed in Cassimeris, 1993; McNally, 1996).

The exact mechanism by which dynamic instability occurs is not known. Originally biochemical analysis of tubulin (Weisenberg et al., 1968) revealed that tubulin is a GTP binding protein. The GTP on β tubulin is hydrolyzable, while the GTP on α tubulin is nonhydrolyzable (MacNeal and Purich, 1978; Spiegelman et al., 1977). Mitchison & Kirschner (1984a) proposed the GTP cap model of dynamic instability, which postulates that a cap of GTP-tubulin at the end of microtubules stabilizes the microtubule and promotes assembly (reviewed in Erickson and O'Brien, 1992; Desai and Mitchison, 1997). This model was based on the work of Carlier and Pantaloni (1981) which suggested that GTP hydrolysis is not coupled to polymerization. As microtubules assemble, GTP-tubulin would be added to the ends of the microtubules forming GTP-tubulin caps. The GTP would be hydrolyzed at a rate independent of the microtubule assembly rate. At equilibrium GTP hydrolysis would overtake assembly in a probabilistic fashion causing the
loss of the GTP cap at an end of some microtubules and rapid disassembly of those microtubules. Other microtubules with GTP caps intact would continue to assemble. Subsequent research has cast doubt on this theory because the ends of microtubules do not disassemble totally after catastrophe as would be predicted by this model (Horio and Hotani, 1986; Walker et al., 1988). Also measurements of the GTP cap indicates that it is very small and that the original data of Carlier and Pantaloni (1981) were incorrect. Studies of tubulin polymerization in the presence of nonhydrolyzable GTP analogs (GMPPNP and GMPPCP) suggest that the primary role of GTP hydrolysis is to destabilize the microtubule lattice by creating GDP-bound subunits that have weaker interdimer contacts (Mandelkow et al., 1991; Vale et al., 1994). More recently Tran et al. (1997a, b) have proposed an updated three-state conformational cap model of microtubule dynamic instability in vitro in which a metastable kinetic intermediate state exists between elongation and shortening states of dynamic instability.

Recently Rodionov and Borisy (1997) have revisited the theory of treadmilling by showing that cytoplasmic microtubules of fish melanophores detach from their nucleation site and depolymerize from their minus ends. These free microtubules are released from centrosomes and move toward the periphery of the cell by treadmilling. Margolis and Wilson (1998) suggest that while microtubules obviously undergo dynamic instability (especially during the interphase to mitotic transition), treadmilling and microtubule motor proteins could account for a poleward migration of microtubules in the mitotic spindle known as flux and for microtubule dynamics of some interphase cells. Interestingly both
dynamic instability and treadmilling may occur in cells, with treadmilling occurring in stable interphase microtubules and in the mitotic spindle causing a "flux" of subunits in the kinetochore to pole direction, while dynamic instability is required for fast rearrangements of microtubules needed for mitosis to occur (reviewed in Anderson, 1998; Margolis and Wilson, 1998).

Microtubule dynamics is also regulated in some cells by proteins called MAPs (microtubule associated proteins). Originally MAPs such as MAP1, MAP2, MAP4 and Tau were identified as proteins that were purified with tubulin when microtubules were purified from mammalian brain tissue by repeated rounds of polymerization and depolymerization (Sloboda et al., 1975; Murphy and Borisy, 1975; Weingarten et al., 1975). MAPs have been shown to stabilize microtubules and promote the assembly of microtubules by binding to the C-terminal region of tubulin (discussed in Nogales et al., 1999) and lowering the critical concentration of tubulin assembly. MAPs also abolish microtubule dynamic instability. They are less prevalent in other tissues than in neuronal tissues, however. So their roles in microtubule dynamics in non-neuronal cells is open to question. For a detailed review of MAPs see Maccioni and Cambiazo (1995).

Microtubules also interact with motor proteins that exist in two main families known as kinesins and dyneins. These motors play an integral role in cellular mechanisms like organelle transport and mitosis (reviewed in Hirokawa et al., 1998; Vallee and Gee (1998).
Tubulin

General characteristics of tubulin

Since the first complete sequences of α- and β-tubulin were published (Krauhs et al., 1981; Ponstingl et al., 1981), analysis of α- and β-tubulin sequences from phylogenetically diverse organisms has shown that all α tubulins are highly homologous to each other and all β tubulins are highly homologous to each other, except for their extreme C-termini. All α tubulins share ≥ 62.3% amino acid identity and all β tubulins share ≥ 63.3% amino acid identity (reviewed in Little and Seehaus, 1988; Burns, 1991; 1994).

Because α and β tubulins from diverse species share high amino acid homology, Burns (1991) postulated that each tubulin monomer has a very similar tertiary structure. Burns’ hypothesis has recently been confirmed by the publication of a 3.7 Å atomic model of the α-β tubulin dimer, obtained by electron crystallography of tubulin sheets polymerized in the presence of zinc (Nogales et al., 1998a). These data reveal that the structure of the two tubulin monomers is nearly identical. Each monomer has been divided into three functional domains based on its structure: 1) the N-terminal domain containing the GTP nucleotide binding region, 2) an intermediate domain, and 3) the C-terminal domain which has been implicated in the binding of MAPs and motor proteins (Nogales et al., 1998a). This model confirms the idea that regions of high homology between α and β tubulin have been conserved because of their functional significance. In addition to its binding GTP and MAPs, the tubulin dimer also binds to medicinally and agriculturally important drugs such as taxol, nocodazole, benomyl, colchicine, and vinblastine (reviewed
in Wilson and Jordan, 1994). Now that the structure of the dimer has been revealed, the sites of action of these drugs may be analyzed in further detail.

α and β tubulin share similar structures with each other and show overall structural similarity to the bacterial cell-division protein FtsZ (Filamenting temperature-sensitive mutant Z), that is the leading candidate for a prokaryotic homolog of tubulin. Even though FtsZ, α, and β tubulin only share 7% sequence homology at the amino acid level their tertiary structures are very similar and the regions of high sequence homology are those involved in GTP binding (Lowe and Amos, 1998; Nogales et al., 1998b). FtsZ also polymerizes into tubular polymers, like tubulin, that form a ring of filaments or tubules needed for septation and proper cell division (Erickson, 1996; Nogales et al., 1998b; Bramhill et al., 1994; Erickson et al., 1995).

Multigene families of α and β tubulins

In 1980, Cleveland and colleagues found that α and β tubulins are encoded by small multigene families using Southern blot analyses of chicken, sea urchin, angler fish, mouse, rat and human genomic DNA. Multiple α- and β-tubulin genes have now been isolated and sequenced from a variety of organisms ranging from ciliated protozoans to humans (reviewed in Little and Seehaus, 1988; Burns et al., 1991). The size of multigene families, within a species, ranges from small families of one or two α- or β-tubulin genes (as in fungi), to large gene families like those found in plants (nine expressed β-tubulin genes in Arabidopsis thaliana) (Raff et al., 1994; Little and Seehaus, 1988). Southern blot analysis suggests that as many as 15-20 α- and β-tubulin sequences may exist in the
human genome (Cleveland et al., 1980). Only four functional α-tubulin genes and four functional β-tubulin genes have been identified in humans while at least nine β-tubulin pseudogenes have been identified (Lee et al., 1983; Wilde et al., 1982; reviewed in Sullivan, 1988; Ludueña, 1998).

The multigene families that encode α- and β-tubulin sequences produce different 'isotypes' of each protein. In this thesis the term 'isotype' will be used to describe different genes and the different polypeptides encoded by these genes. The term 'isoform' will be used to describe an α- or β-tubulin protein that has been post-translationally modified, and they will be discussed later in this chapter. Multiple α- and β-tubulin isotypes have been identified in protists, fungi, plants and animals.

The 15 C-terminal amino acids of α and β tubulins are highly variable. When sequences of avian and mammalian β-tubulins were compared however, it was found that isotype classes of tubulins exist. The 15 C-terminal amino acids of tubulins within an isotype class are conserved among organisms but they differ as much as 100% with tubulins of other isotype classes, even within the same organism. Vertebrate β-tubulin genes fall into six major isotype classes (Sullivan and Cleveland, 1986; Little and Seehaus, 1988; Ludueña, 1988).

Within a given isotype class, the 3' noncoding regions of α and β tubulins are often conserved across species (reviewed in Little and Seehaus, 1988; Cleveland and Sullivan, 1986). The same is true for the 5' untranslated region of tubulins but to a lesser degree (Cowan and Dudley, 1983; Little and Seehaus, 1988). The conservation of vertebrate
isotype classes suggests that different isotypes may have functional significance. Unlike
the sequences of α and β tubulins in higher eukaryotes, α- and β-tubulin sequences in
lower non-animal eukaryotes (i.e. fungi and protists) are postulated to have diverged from
other tubulin sequences because of fewer structural constraints on tubulins in these
organisms (Raff et al., 1994; Ludueña, 1998). α-Tubulin genes also fall into six major
isotype classes. These isotype classes are conserved in mammals but are not conserved in
birds.

Another level of tubulin heterogeneity is due to post-translational modification of
tubulin. Post-translational modifications may be the only sources of tubulin diversity in
species that lack multiple tubulin isotypes (e.g. Chlamydomonas reinhardtii, Youngblom et
al., 1984). These modifications occur on either monomer or on both, as well as the
microtubule polymer itself and they are enzymatically driven. α-tubulin modifications
include acetylation and the detyrosination and tyrosination of the C-terminus (reviewed in
MacRae, 1997; Ludueña, 1998). A special form of α-tubulin called Δ2-tubulin found in
cells involved in neuronal processes exists as a result of the removal of the C-terminal
tyrosine and glutamine (Mary et al., 1996; MacRae, 1997). β-tubulins are modified by
phosphorylation of residues like tyrosine and serine (MacRae, 1997). Both α and β
tubulin undergo polyglutamylation or polyglycylation (Edde et al., 1990; Redicker et al.,
1994). All these modifications seem to be associated with tubulin in stable microtubule
populations like those found in axonemes, and brains and with subpopulations of stable
microtubules found in the cytoplasm of mammalian cells.
**Functional significance of multiple tubulin isotypes**

Although it has been known for almost twenty years that tubulins are encoded by multigene families, the functional significance of multiple tubulin isotypes is still being debated today. Prior to the identification of tubulin as a heterodimer and prior to the cloning of multiple tubulin genes, Behnke and Forer (1967) were the first to propose that different tubulins are found in different microtubular organelles of cranefly spermatids based on the different biochemical properties of the microtubules. Subsequently, Fulton and Simpson (1976) proposed the "multi-tubulin hypothesis" which states that an organism has multiple tubulin genes that encode different polypeptides which support different microtubule functions. To date the only evidence that substantiates this theory has been found with a *D. melanogaster* testis-specific β2 gene (Hoyle and Raff, 1990, Fackenthal et al., 1995; Raff et al., 1997) and very specialized α- and β-tubulin isotypes found in *Caenorhabditis elegans* (Savage et al., 1989; Fukushige et al., 1999). Consequently, Raff (1994) proposed that multiple tubulin genes may provide an organism with a mechanism to regulate gene expression both temporally and spatially, while the expression of multiple tubulin isotypes in a single cell may ensure that a sufficient pool of tubulin is expressed in the cell.

When considering the significance of the expression or existence of multiple tubulin isotypes Ludueña (1998) suggested that there are three basic models to consider: (1) isotypes have no functional significance; (2) isotypes are adaptive but do not perform specific functions; and (3) isotypes perform specific functions. Examples that support each
of these models exist in nature.

The first model postulates that isotypes have no inherent functional significance. For example, although mammalian β tubulins exist in six isotype classes that clearly are differentially expressed, all the cytoplasmic and mitotic microtubules in mammalian cells are mixed copolymers of all of the expressed β-tubulin isotypes (Lewis et al., 1987; Lopata and Cleveland, 1987; Lewis and Cowan, 1988). This suggests that these isotypes are interchangeable and do not affect microtubule assembly or other characteristics. Many other examples of interchangeability of isotypes exists (Schatz et al., 1986; Gu et al., 1988; May, 1989; Kirk and Morris, 1993).

According to the second model, having a large pool of tubulin isotypes allows an organism to adapt to environmental challenges but the isotypes are not preadapted to perform specific functions. For instance, in A. thaliana, transcription of different β-tubulin genes may be temperature dependent. Transcription of β-tubulin isotypes TUB2, TUB3, TUB6, and TUB8 have decreases in transcription levels at low temperatures while TUB9's transcription level increases. Ludueña (1998) suggests that these plants are using their isotypes to adapt to environmental factors. There are many examples of isotype adaptability to drugs and extreme temperatures in plants and fungi (Modig et al., 1999; Yan and Dickman, 1996; Chu et al., 1993; Ranganathan et al., 1998; Sangrajrang et al., 1998). Interestingly, however, fungi and some other lower eukaryotes are able to adapt to a variety of environmental factors although they only have one or two α- or β-tubulin genes.

The third model postulates that different isotypes are required for different
microtubule functions. The strongest argument supporting this model is the high conservation of isotype differences over evolutionary time and the conservation of isotype classes across species. It has been suggested that constraints on isotype divergence resulted because of the essential functions microtubules provide to cells (Raff et al., 1994). Savage et al. (1989) were some of the first researchers to show that a specific β-tubulin isotype (mec-7) in *C. elegans* is required for the production of 15 protofilament microtubules in axons of touch receptor neurons. More recently, Fukushige et al. (1999) have isolated another gene, Mec-12, an α-tubulin isotype in *C. elegans*, that is required along with Mec-7 to assemble the proper 15-protofilament microtubules needed in the axons of touch receptor neurons. Therefore, in this case, the assembly of a specific type of microtubule requires very specific α- and β-tubulin isotypes to be functional.

Experiments done over the past 18 years in the Raff lab have also advanced the notion that some tubulin isotypes have specific functions. Hoyle and Raff (1990) showed that a divergent, developmentally regulated *D. melanogaster* β3 isotype cannot support the specific axonemal functions of the testis-specific β2 isotype in β2 null flies. More recently, Raff et al. (1997) wanted to determine if an orthologous β2 isotype from a moth is functionally equivalent to the *D. melanogaster* β2 isotype. The moth β2 isotype was unable to support any microtubule assembly in a *D. melanogaster* β2 null background. Coexpression of the moth β2 at greater than 6% of the total tubulin pool results in accessory axonemal microtubules composed of 16 protofilaments (like those normally found in moths) instead of the normal 13 protofilament microtubules found in *D.*
melanogaster (Raff et al., 1997). This finding along with the data on Mec-12 and Mec-7 in *C. elegans* suggests that protofilament number and microtubule architecture may be regulated by specific tubulin isotypes. Consequently, Wilson and Borisy (1997) have suggested that tubulins provide isotype-specific functions in specialized cells.

**Microtubule organizing centers**

In the late 1800's cytologist Theodor Boveri observed a cellular structure at the center of the mitotic spindle apparatus in *Ascaris* eggs and he named this structure the "centrosome" which means "central body" (Wilson, 1925). Later, using electron microscopy, Porter (1966) was able to show that microtubules are organized around centrosomes. Pickett-Heaps (1969) coined a more general name "microtubule organizing center" (MTOC) to refer to the morphologically diverse centers that share the ability to nucleate and organize microtubules. Although it has been shown that microtubules have the ability to self-assemble, microtubule assembly is often nucleated by MTOCs in vivo (Mitchison and Kirschner, 1984b; Desai and Mitchison, 1997). The structure of MTOCs varies widely from cell to cell and organism to organism. They include the centrosomes of higher eukaryotes, the spindle pole bodies (spb) of fungi, and blepharoplasts of lower plants. Interestingly, in higher plants there is no morphologically defined structure that acts as an MTOC.

While the primary function of MTOCs is to organize microtubules by nucleating microtubule assembly at a concentration of tubulin below the critical concentration of
assembly, MTOCs also appear to establish the protofilament number of nucleated microtubules (Evans et al. 1985). MTOCs also establish the orientation of the microtubules within cells by nucleating microtubules with their minus ends proximal to and plus ends distal from the MTOCs (McIntosh and Eutener, 1984). MTOCs organize microtubules into polar arrays that are essential for the directed movement of organelles along microtubules powered by members of the kinesin and dynein families of motor proteins (Ault and Rieder, 1994; Goldstein and Vale, 1991; Vallee, 1991). Kinesins are mostly plus-end directed motors that transport proteins (such as proteins needed in synaptic membranes at the end of axons) and organelles such as vesicles away from the MTOC (reviewed in Hirokawa et al., 1998). Conversely, dynein is a minus-end directed motor that transports certain proteins and vesicles toward the MTOC which is usually located near the nucleus (reviewed in Vallee and Gee, 1998). Both families of proteins may be involved in movement of chromosomes along kinetochore fibers during mitosis.

**Centrosomes**

As stated previously, the centrosomes of animals have been studied for more than 100 years. Centrosomes are small organelles, approximately 1 micron in diameter, which are composed of a pair of centrioles that lie perpendicular to each other and are surrounded by an amorphous electron-dense matrix called the pericentriolar material (PCM) (reviewed in Doxsey, 1998; Stearns and Winey, 1997; Balczon, 1996). Centrioles are cylindrical structures composed of nine short microtubule triplets (reviewed by Rose et al., 1993; Kuriyama and Kanatani, 1981). The centrosome is anchored to the surface of the nuclear
envelope in interphase cells (Kellogg et al., 1994). Its location in the cell is important because the centrosome's function in the cell depends on its location during interphase (at the cell's center) and during mitosis (at each pole). Centrosomes have three basic functions: (1) they nucleate the polymerization of microtubules, (2) they organize microtubules into functional arrays, and (3) they duplicate once every cell cycle (Urbani and Stearns, 1999; Stearns and Winey, 1997).

Centrosome duplication occurs once every cell cycle starting at the G1/S transition and ending at the start of mitosis (Kellogg et al., 1994). During duplication a 'daughter' centriole forms adjacent to, and perpendicular to, each pre-existing centriole (reviewed in Marshall and Rosenbaum, 1999). The 'mother' or older centriole in the pair has spoke-like appendages near its distal end and the two centrioles are connected by fibers at their proximal ends (reviewed in Urbani and Stearns, 1999). Cenexin, a 96 kd protein identified by monoclonal antibodies (mAbs) to mammalian centrosomal immunogens, is a marker of centriole maturation acquired at the G2/M transition by the immature centriole (Lange and Gull, 1995). Until recently the molecular controls of duplication remained an enigma; however, recently the cdk2-cyclinE complex has been identified as the regulator or trigger for centrosome duplication (Hinchcliffe et al., 1999; Lacey et al., 1999; reviewed in Pennisi, 1999). A new gene, UNI3, has been cloned and shown to be required for the addition of the third microtubule of the triplet to the centriole. This gene encodes a new member of the tubulin superfamily of proteins, δ tubulin (Dutcher et al., 1998).

While the centrosome has been viewed as the preferred site of microtubule
assembly *in vivo* for a long time (Brinkley, 1985), it was Gould and Borisy (1977) who found that the PCM is the actual site of microtubule nucleation at centrosomes. This discovery led cell biologists to ask two questions that they have continued to investigate until the present. What is the primary function of centrioles since they do not actually nucleate microtubule assembly, and what specific components of the PCM actually nucleate microtubule assembly? Recently Marshall and Rosenbaum (1999) have suggested that centrioles may act as organizing centers that provide a scaffold around which microtubule nucleating factors located in the PCM are recruited into a single focus, forming the centrosome. Bobinnec *et al.* (1998) have reported that centrioles are needed to organize pericentriolar components into a structurally stable organelle. Centrioles also seem to be needed to assemble a functional centrosome that can nucleate astral microtubules which provide proper nuclear spacing and migration during syncytial divisions in *Sciara* (de Saint Phalle *et al.*, 1998).

Because the centrosome is a complex organelle that is contiguous with the cytoplasm of the cell (unlike membrane-bound organelles) it has been difficult to isolate and identify the protein components of the PCM. Biochemical and genetic analysis of centrosomes has shown that over 100 proteins localize to the centrosome during various stages in the cell cycle (reviewed in Kalt and Schliwa, 1993; Rose *et al*., 1993; Kellogg *et al*., 1994; Balczon, 1996). Caution must be taken before assuming these proteins are functional components of the centrosome that are involved in its nucleating ability. Some proteins may localize to the PCM because they are the cargo that is attached to minus-end
directed motors that travel toward the PCM along microtubules; others may be deposited at the centrosome prior to mitosis to ensure that they are partitioned to both cells when cytokinesis occurs. In essence, it is likely that the centrosome is composed of functional proteins involved in nucleation and regulatory proteins that are involved in several cellular processes that are under cell cycle control.

Initially, putative components of the centrosome were identified using polyclonal or monoclonal antibodies, while some researchers characterized the centrosome using standard biochemical purification procedures (reviewed in Kimble and Kuriyama, 1992; Kalt and Schliwa, 1993). Many centrosomal components have been categorized based on their localization patterns during the cell cycle. Some components are detectable at the centrosome throughout the cell cycle, while others are only detectable during mitosis. There are also proteins that localize to the centrosome during mitosis but are found in other areas of the cell during the remainder of the cell cycle.

Cytocentrin and centrosomin are two proteins that localize to the centrosome during mitosis but are found in the cytosol during interphase. Cytocentrin is a Ral-GTPase binding protein that seems to be involved in the separation of centrosomes during mitosis in rat cell lines (Quaroni and Paul, 1999; Paul and Quaroni, 1993). Centrosomin was identified as a regulatory target of homeotic genes. It has been shown to be essential for the assembly and function of centrosomes during syncytial divisions in D. melanogaster (Megraw et al., 1999; Li and Kaufman, 1996). Since homologs to neither protein have been characterized in other organisms, their overall functional significance is as yet
Several proteins are located at the centrosome during mitosis and in other noncytosolic cellular locations during interphase. In *D. melanogaster*, Raff *et al.* (1993) identified DMAP60 and DMAP190, two proteins that localize to the centrosome during mitosis and have a microtubule binding capacity. During interphase these two proteins localize to the nucleus (Raff *et al.*, 1993). The function of these proteins remains unknown, but neither seems to play a direct role in nucleation of microtubules (Oegema *et al.*, 1999).

One of the proteins most recently found to localize to the centrosome during mitosis and the nucleus during interphase is BRCA1, a suppressor of tumorigenesis in breast and ovarian cells (Hsu and White, 1998). BRCA1 coimmunoprecipitates with γ-tubulin and its function at the centrosome will be discussed in more detail in the γ-tubulin section of this introduction.

Centrin, pericentrin, and γ-tubulin are some of the proteins that reside at the centrosome throughout the cell cycle. Centrin, a member of the EF-hand superfamily of Ca\(^+\) binding proteins, has been localized to the MTOCs of plants, the SPBs of yeast and to animal centrosomes. Although two isotypes of centrin have been cloned in humans (Errabolu *et al.*, 1994; Lee and Huang, 1993), its functional significance is still unknown. Centrin is involved in SPB duplication in yeast and flagellar severing in *Chlamydomonas reinhardtii* indicating it has different functions in different organisms. A common molecular mechanism, such as the contraction of EF-hand proteins upon binding to Ca\(^+\),
may underlie its functions (Schiebel and Bornens, 1995; Balczon, 1996). Pericentrin, a 220 kd coiled-coil protein that localizes to the PCM of *X. laevis* embryos, is required for proper formation of microtubule asters around centrioles and may provide scaffolding upon which \( \gamma \) tubulin and its associated proteins nucleate microtubule assembly (Doxsey *et al.*, 1994; Dictenburg *et al.*, 1998).

\( \gamma \) tubulin, the third member of the tubulin superfamily (Oakley and Oakley, 1989), is required for the nucleation of microtubules at centrosomes and is located at the MTOC throughout the cell cycle. The details of its function will be discussed in the last section of this introduction.

**Spindle Pole Bodies**

The spindle pole body (SPB) serves as the MTOC in fungi and is the functional homolog of the animal centrosome. The SPB of *Saccharomyces cerevisiae* is the most extensively analyzed model of MTOC function because researchers have been able to use the power of yeast genetics and molecular genetics to analyze SPB components. The SPB has been shown to nucleate microtubules *in vivo* and *in vitro*, verifying that it is the MTOC of yeast (Byers *et al.*, 1978; Hyams and Borisy, 1978). Byers and Goetsch (1975) used electron microscopy to analyze the substructure of SPBs and found that the SPB is embedded in the nuclear envelope throughout the cell cycle. SPBs have a trilaminar morphology consisting of a central plaque that is coplanar with the nuclear envelope, and an inner plaque and an outer plaque. More recently, Bullitt *et al.* (1997) and O'Toole *et al.* (1999) have characterized the fine structure of frozen SPBs using electron tomography and
have determined that the trilaminar SPB is actually composed of six distinct layers.

Over the past ten years several components of the yeast SPB have been identified via genetic screens for mutations in SPB components or biochemical analysis of isolated SPBs. The Kilmartin lab was the first lab to biochemically characterize SPB components biochemically (Rout and Kilmartin, 1990) and recently has used high-mass accuracy matrix-assisted laser desorption/ionization (MALDI) mass spectrometry mapping in combination with sequence database searching to identify 11 new gene products that localize to the SPB and 12 previously identified SPB components (Wigge et al., 1998). For an in-depth review of SPB components see Wigge et al. (1999), and Elliot et al. (1999).

Spc110p was one of the first proteins identified by Rout and Kilmartin (1990; Kilmartin et al., 1993). It has been studied extensively and found to localize to the nuclear side of the SPB where it links or anchors the central plaque to the inner plaque by way of its central coiled-coil protein domain. More recently the N-terminal region of Spc110p has been shown to mediate the binding of Spc98p and Spc97p, two other SPB components, to the inner plaque of the SPB via a direct interaction with Spc98p (Knop and Schiebel, 1997b; Sunberg and Davis, 1997; Nguyen et al., 1998). Spc98p and Spc97p bind to Tub4p, the yeast γ tubulin, forming the Tub4p complex. Another SPB component, Spc72p, has been shown to tether the Tub4p complex to the cytoplasmic side of the SPB by interacting directly with Spc98p via its N-terminus (Knop and Schiebel, 1998; reviewed in Saunders, 1999) in a manner analogous to Spc110p.
Homologs of Spc110p, Spc98p, Spc97p have been found in various animals further substantiating the theory that some SPB components are general components of MTOCs.

**Plant MTOCs**

Lower land plants, like bryophytes and pteridophytes have structurally defined MTOCs in some stages of their development (Marc, 1997; Vaughn and Harper, 1998), especially in those plants which have flagellated cells. Some of the discrete MTOCs of lower plants include bicentrioles (two centrioles joined end to end), polar organizers (an extension of the nuclear envelope), blepharoplasts (large spherical templates for centrioles), and a multilayered structure (a trilaminar strip of reorganized PCM) (reviewed in Vaughn and Harper, 1998).

In higher plants five different microtubule arrays form during the cell cycle: The interphase radial cytoplasmic array; the cortical array (which forms during interphase); the preprophase band (seen in G2 and early mitosis); the barrel-like acenrtioral bipolar mitotic spindle; and the phragmoplast (Balczon, 1996; Vaughn and Harper, 1998).

Since plants do not have morphologically discrete MTOCs, the existence of homologs of common SPB or centrosomal nucleating elements in plants may indicate that plants have flexible MTOCs like those proposed by Mazia (1987). In recent years several centrosomal components have been localized to plants in areas where microtubule arrays are nucleated. These proteins include centrin, pericentrin and γ-tubulin.

For many researchers the cloning of γ-tubulin cDNAs from several plant genomes
(e.g. Allium cepia, Anemia phylitidis, Arabidopsis thaliana) has confirmed that plants, like animals and fungi, have MTOCs that nucleate microtubule assembly, although they are not morphologically discrete (McDonald et al., 1993; Liu et al., 1994; Marc, 1997).

\textbf{γ Tubulin}

**Discovery of γ tubulin**

In a search for genes whose protein products interact with microtubules, Weil et al. (1986) identified two new loci, designated \textit{mipA} and \textit{mipB} (microtubule-interacting proteins A and B), in Aspergillus nidulans. The \textit{mip} loci were found in a genetic screen for revertants of the \textit{A. nidulans} β-tubulin mutant allele \textit{benA33}. The \textit{benA33} allele confers heat sensitivity and seems to hyperstabilize microtubules at restrictive temperatures (Oakley and Morris, 1981). Revertants of \textit{benA33} suppressed its heat-sensitive phenotype and in some cases conferred cold sensitivity. Three alleles at the \textit{mipA} locus conferred allele-specific synthetic phenotypes in combination with various \textit{benA} alleles.

The \textit{mipA} gene product was cloned and sequenced by Oakley and Oakley (1989) and it encoded a novel member of the tubulin superfamily that they named \textit{γ} tubulin. Disruption of the \textit{mipA} gene blocks nuclear division and disrupts assembly of the mitotic apparatus, indicating that \textit{γ} tubulin is an essential gene that is necessary for the nucleation of microtubule assembly (Oakley et al., 1990). Further analysis of \textit{γ} tubulin using polyclonal antibodies revealed that \textit{γ} tubulin is a component of the SPB of interphase and mitotic cells in \textit{A. nidulans} and of the centrosomes of HeLa and mouse (3T6) cell lines.
These findings led Oakley and colleagues (1990; Zheng et al., 1991a, b) to propose that γ tubulin nucleates microtubule assembly at MTOCs, attaches microtubules to MTOCs and establishes microtubule polarity in vivo.

Ubiquity of γ tubulin

Subsequent to the initial identification of γ tubulin in A. nidulans, Zheng et al. (1991b) cloned γ-tubulin cDNAs from D. melanogaster and Hela cell cDNA libraries, thus demonstrating that γ tubulin is probably a ubiquitous protein in eukaryotes. These two γ-tubulin cDNAs share 66.7% and 66.4% amino acid identity with the A. nidulans γ-tubulin gene respectively. To date γ-tubulin genes or cDNAs have been identified in 36 species (see Table 1) ranging from plants to fungi to humans (reviewed in Burns, 1995a). All of these γ-tubulin sequences are highly conserved sharing at least 66% amino acid identity except for C. elegans, S. cerevisiae (TUB4) and Candida albicans γ-tubulin genes which share approximately 44%, 36%, and 40% amino acid identity respectively with the other species identified to date (Burns, 1995b; Keeling and Doolittle, 1996; Akashi, 1999). Although the S. cerevisiae γ-tubulin is quite divergent it is essential to the microtubule nucleating capacity of the SPB (Spang et al., 1996; Marschall et al., 1996). Neither the human γ-tubulin (Hγ1) cDNA nor the Xenopus γ-tubulin cDNA complements a mutant TUB4 allele indicating that these proteins are not interchangeable (Spang et al., 1996; Marschall et al., 1996). Marschall et al. (1996) also reported that TUB4 does not complement a S. pombe γ-tubulin null mutation. The γ tubulin homologs in C. elegans and S. cerevisiae may be highly divergent from other γ-tubulin genes because they have
specialized functions in these organisms (reviewed in Oakley, 1999).

Sequence analysis of a diverse range of organisms reveals that $\gamma$ and $\beta$ tubulins share 35-36% amino acid identity, while $\gamma$ and $\alpha$ tubulins share 33-35% amino acid identity (reviewed in Burns, 1995a). The N-terminal region of $\gamma$ tubulin (5' to amino acid 276) is more homologous to the amino acids of $\alpha$ and $\beta$ tubulin than is the C-terminal region. Because the sequences of $\alpha$, $\beta$, and $\gamma$ tubulins are similar they are likely to have similar secondary and tertiary structures (Burns, 1994; 1995a). Many of the most highly conserved regions shared between $\alpha$, $\beta$, and $\gamma$ tubulins correspond to regions of the tubulin dimer that have been shown to bind GTP (Burns, 1995a; Nogales et al., 1998a; 1999).

Therefore, $\gamma$ tubulin was hypothesized to be a GTP binding protein like $\alpha$ and $\beta$ tubulin. Melki et al. (1993) showed that $\gamma$ tubulin preferentially binds to columns of immobilized GTP rather than ATP and Oegema et al. (1999) showed that the $\gamma$ tubulin found in a small $\gamma$ tubulin complex of *Drosophila* preferentially binds to GDP.

**Cellular localization and function**

$\gamma$ Tubulin has now been localized to various types of MTOCs in several plants and animals, and fungi (reviewed in Burns, 1995a; Pereira and Schiebel, 1997). $\gamma$ Tubulin has also been localized to the basal bodies of specialized mammalian cells, ciliated protozoans, and lower plants (Muresan et al., 1993; Fuchs et al., 1993; Liang et al., 1996; Ruiz et al., 1999; Silflow et al., 1999). Interestingly, acentriolar MTOCs of certain animal and plant cell types also contain $\gamma$ tubulin (McDonald et al., 1993; Julian et al., 1993; Gueth-hallonet
<table>
<thead>
<tr>
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<td>Zheng, Jung &amp; Oakley (1991)</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>Zheng, Jung &amp; Oakley (1991)</td>
</tr>
<tr>
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<td>Horio et al. (1991)</td>
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<td>Xenopus laevis</td>
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<td>Yarrowia lipolytica</td>
<td>Stearns et al. (1991)</td>
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<td>Mus musculus</td>
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<td>Ustilago violacea</td>
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<td>Akkari et al. (1994)</td>
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<td>Chlamydomonas reinhardtii</td>
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<td>Oakley &amp; Fong (unpub.)</td>
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<td>Scott et al. (1997)</td>
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<td>Entamoeba histolytica</td>
<td>Lohia &amp; Samuelson (1997)</td>
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<td>Microbotryum violaceum</td>
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<tr>
<td>Candida albicans</td>
<td>Akashi et al. (Genbank)</td>
</tr>
<tr>
<td>Rattus norvegicus</td>
<td>Nakadai et al. (1999)</td>
</tr>
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</table>

Table 1: Organisms from which γ-tubulin genes have been isolated.
et al., 1993; Palacios and Joshi, 1993; Gard, 1994a; Debec et al., 1995; Endow and Komma, 1998).

γ tubulin localizes along the length of kinetochore microtubules of several plant cells during mitosis, but it is not found at the kinetochore (Liu et al., 1993; McDonald et al., 1993). Lajoie-Mazenc and colleagues (1994) observed γ-tubulin staining at centrosomes and in the mitotic spindle of several mammalian tissue culture cell lines. Immunoelectron microscopy data using these same cell lines suggests that γ tubulin may associate with the walls of spindle microtubules (Lajoie-Mazenc et al., 1994). This localization pattern might also be due to the presence of the staggered minus ends of microtubules found throughout the spindle length (Liu et al., 1994). Endow and Komma (1998) have shown that γ-tubulin (γTUB37CD) also localizes to a central spindle pole found in meiosis II D. melanogaster oocytes. Localization of γ tubulin to this central pole is dependent on the NCD minus-end directed motor protein. Wilson and Borisy (1998), however, did not observe γTUB37CD at the meiosis II central spindle pole. The cause for the discrepancies in these two papers is not clear.

It is evident that γ tubulin's localization to various types of MTOCs is linked to its function as a nucleator of microtubule assembly, but γ tubulin may also function in other capacities as well. Several labs have independently localized γ tubulin to the barrel of centrioles of mammalian cells and the basal bodies of Chlamydomonas reinhardtii and Paramecium tetraurelia (Dibbayawan et al., 1995; Fuller et al., 1995; Moudjou et al., 1996; Ruiz et al., 1999; reviewed in Marschall and Rosenbaum, 1999). Ruiz et al. (1999) have
shown that inactivation of γ-tubulin genes in *P. tetraurelia* inhibits basal body duplication, indicating that γ tubulin is required for basal body duplication. These findings support the work of Fuller *et al.* (1995) who proposed that γ tubulin is located on the outer walls of parental centrioles and acts as a template for daughter centriole formation.

Oakley's original hypothesis that γ tubulin nucleates microtubule assembly *in vivo* at MTOCs (Oakley *et al.*, 1990) has been substantiated by several studies. Horio *et al.* (1991) and Stearns *et al.* (1991) independently disrupted the γ-tubulin gene in *Schizosaccharomyces pombe* and found that γ tubulin is essential for cell viability and nuclear division. Plasmid loss experiments, in which a multicopy plasmid containing the *S. pombe* γ-tubulin gene is lost from into a haploid strain containing a disrupted γ-tubulin gene, suggest that γ tubulin is necessary for proper spindle structure and function (Horio *et al.*, 1991). Later, Joshi (1992) demonstrated that anti-γ-tubulin antibodies injected into mammalian cells inhibited cytoplasmic microtubule reassembly and the formation of functional mitotic spindles. γ-tubulin function is also conserved in phylogenetically distant organisms as was shown when the human γ-tubulin cDNA (Hy1) restored viability to *S. pombe* cells that lacked endogenous γ tubulin (Horio and Oakley, 1994). Characterization of a mutant allele of *D. melanogaster* γ tubulin (γTUB23CD) revealed that γ tubulin is not only required for proper spindle function but it is also apparently required for proper MTOC structure because the mutation results in the formation of abnormal MTOCs although microtubule assembly still occurs in these mutants (Sunkel *et al.*, 1995).

Recently, Khodjakov and Rieder (1999) have reported the use of a γ-tubulin green
fluorescent protein (γTGFp) in immunofluorescence studies to determine how γ tubulin behaves during the cell cycle in mammalian cell lines. They show that γTGFp exists at low levels at the centrosome during interphase, but the amount of γ tubulin increases by four fold at the onset of mitosis. γTGFp migrates into the spindle during mitosis, corroborating earlier findings by Lajoie-Mazenc et al. (1994) and others. Two populations of γTGFp exist at the centrosome; one population rapidly exchanges with soluble γ tubulin, while another population seems to be more stably bound to the centrosome and exchanges slowly with soluble γ tubulin. The centrosome appears to become activated at the onset of mitosis to bind increased amounts of γTGFp.

Martin et al. (1997) analyzed the mipA (γ-tubulin) gene disruption phenotype in A. nidulans conidia that were blocked in G2 and released. Results of this analysis indicated that γ tubulin is absolutely required for nucleation of the mitotic spindle and nuclear division. The absence of functional γ tubulin causes a transient mitotic block. γ Tubulin is not required, however, for cell cycle progression from G1 to M nor for SPB duplication in A. nidulans.

Biochemical characterization of γ tubulin and the γTuRC

γ tubulin is a minor cellular protein (in amount). It comprises only 0.01% of the cell’s total protein in unfertilized Xenopus eggs (Stearns et al., 1991). This makes it difficult to purify γ tubulin from cells. Since it is a minor protein, researchers tried to produce γ tubulin in vitro as a fusion protein in E. coli, but like α and β tubulin, γ tubulin forms insoluble inclusion bodies (Melki et al., 1993). γ tubulin, like α and β tubulin, is
folded by TCP1 chaperonin complexes prior to its release into the cytoplasm in a soluble form (Melki et al., 1993). Unfolded or improperly folded γ tubulin binds to prefoldin, a chaperone that forms a complex with TCP1 where γ is folded into its native form (Vainberg et al., 1998). The absence of a TCP1 complex in E. coli may preclude correct folding.

Subsequently, biochemical analysis of γ tubulin in Xenopus laevis oocyte cell-free extracts revealed that γ tubulin is recruited from the oocyte cytoplasm in order to activate the sperm centriole and form an active centrosome in vitro (Félix et al., 1994; Stearns and Kirschner, 1994). Stearns and Kirschner (1994) found that cytoplasmic γ tubulin sediments as a 25S complex they named the γ-some. Zheng et al. (1995) developed a procedure using antibody-affinity chromatography to isolate this 25S γ-some complex from Xenopus egg extracts. Analysis of this complex showed that it contains γ tubulin and at least six other proteins including α and β tubulin (Zheng et al., 1995). Electron microscopy analysis revealed that the complex is a 25 nm ring structure that was re-named the γ-TuRC (γ tubulin ring complex). The complex nucleates microtubule assembly and binds to the minus ends of microtubules in vitro (Zheng et al., 1995). Immuno-electron microscopic tomography was used by Moritz et al. (1995) to localize γ tubulin to 25-30 nm ring structures in the PCM of purified D. melanogaster centrosomes. Therefore γTuRCs probably reside in the PCM where they nucleate microtubules at their minus ends thereby establishing the polarity of the microtubules. Subsequently, Vogel et al. (1997) reported that the PCM of Spisula solidissima oocyte centrosomes contain ring-shaped structures that are approximately 25 nm in diameter, like those seen by Moritz et al. (1995). More
recently, Moritz et al. (1998) have shown that D. melanogaster salt-stripped centromosomal scaffolds lack the ability to nucleate microtubule assembly. When D. melanogaster embryo extracts are added to the salt-stripped centrosomes, they regain the ability to nucleate microtubule assembly. If γ-TuRCs are immunodepleted from the extracts, microtubules nucleation is not restored. If purified γ-TuRCs and an as yet unidentified 220 kd protein are added to salt-stripped centrosomes microtubule nucleation is restored. Similarly, Schnackenberg et al. (1998) were able to reconstitute salt-stripped, inactivated centrosomes isolated from S. solidissima using S. solidissima oocyte extracts which contain γ tubulin and 25 nm ring structures. All these data support the original hypothesis of Oakley et al. (1990) that γ tubulin nucleates microtubule assembly at MTOCs. Li and Joshi (1995) also reported that γ-tubulin binds to the minus ends of microtubules of axonemes in vitro further supporting another portion of Oakley’s hypothesis (Oakley et al., 1990).

As a consequence of the identification of γ-TuRCs in X. laevis, other researchers have searched for cytoplasmic γ-tubulin complexes in various organisms and have begun to characterize the γ-TuRC subunits (reviewed in Wiese and Zheng, 1999). Components of γ-TuRCs, called gamma tubulin ring proteins (grips), have been identified in D. melanogaster, mouse 3T3 cells, humans, sheep, A. nidulans and S. cerevisiae (Moritz et al., 1998; Oegema et al., 1999; Murphy et al., 1998; Tassin et al., 1998; Detraves et al., 1997; Akashi et al., 1997; Knop and Schiebel, 1997a, b). These components are listed in Table 2 (adapted from Wiese and Zheng, 1999) with homologous proteins located on the
same row. The \textit{S. cerevisiae} \(\gamma\)-tubulin protein, Tub4p, is found in a 6S complex that contains two molecules of Tub4p and one molecule each of Spc98p and Spc97p (Knop and Schiebel, 1997b). This complex is required for the nucleation of microtubule assembly from both the cytoplasmic and nuclear sides of the SPB and it is tethered to the nuclear side of the SPB by SPC110p (Geissler et al., 1996; Knob and Schiebel, 1997a, b). Oegema \textit{et al.} (1999) have isolated a complex from \textit{D. melanogaster}, the \(\gamma\)-tubulin small complex (\(\gamma\)-TuSC) that appears to be analogous to the Tub4p complex found in \textit{S. cerevisiae}. \(\gamma\)-TuSC contains homologs of Spc97p and Spc98p that they have named Dgrip84 and Dgrip91 (Oegema \textit{et al.}, 1999). Spc97p and Spc98p homologs have been identified in \textit{D. melanogaster}, \textit{X. laevis}, and \textit{H. sapiens} (Martin \textit{et al.}, 1998; Murphy \textit{et al.}, 1998; Oegema \textit{et al.}, 1999; Tassin \textit{et al.}, 1998). Spc97p and Spc98p are related and may be members of a protein superfamily.

Dictenberg and colleagues (1998) reported that pericentrin, a 220 kd protein that localizes to the PCM, and \(\gamma\) tubulin are a part of a large complex that forms a centrosomal lattice that is conserved from mammals to amphibians. Since pericentrin has been predicted to be a coiled-coil protein it may form the base of a centrosomal lattice that organizes \(\gamma\) TuRCs at the centrosome where they nucleate microtubules (Doxsey \textit{et al.}, 1994; Dictenberg \textit{et al.}, 1998). As mentioned previously, Moritz and colleagues (1998) have shown that \(\gamma\) TuRC and an unknown 220 kd protein are required to make salt-stripped centrosomal scaffolds competent to nucleate microtubule assembly in \textit{D. melanogaster}. Recently the Asp protein, a 220 kd product of the Asp (abnormal spindle) gene in \textit{D.
*melanogaster*, has been implicated as the protein that restores microtubule nucleating activity to these salt-stripped centrosome preparations in the presence of γTuRC (de Carmo Avides and Glover, 1999). Asp seems to be required to focus the γTuRC within the PCM so it can nucleate asters of microtubules (de Carmo Avides and Glover, 1999). Since Asp does not copurify with γTuRC, Asp is probably not directly involved in microtubule nucleation; purified γTuRCs can nucleate microtubules but microtubules are not organized in discrete centers or asters unless Asp is present. The functions of pericentrin and Asp seem to overlap and they may be functional homologs.

An unexpected recent finding is the localization of the BRCA1 protein to centrosomes during mitosis and its coimmunoprecipitation with γ-tubulin (Hsu and White, 1998). The BRCA1 gene encodes a 1863 amino acid (aa) protein that is a suppressor of tumorigenesis in breast and ovary tissues (O'Brien *et al.*, 1994; Wooster *et al.*, 1994). BRCA1 localizes to the nucleus during interphase and it is phosphorylated during late G1 and S phase (Xu *et al.*, 1999). A hypophosphorylated form of BRCA1 has been immunoprecipitated with γ tubulin. Hsu and White (1998) have proposed that BRCA1 may be a regulator of mitotic spindle assembly and of the G2/M transition. Xu *et al.* (1999) reported that a mutant BRCA1 gene expressed in mouse embryonic fibroblasts causes the G2/M checkpoint control to be defective and 25% of the mutant cells have amplified, functional centrosomes that form multiple spindle poles in each cell. Taken together these results link the centrosome's microtubule nucleating capacity and the control of cell cycle progression.
**Organisms:**

<table>
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<tr>
<th>Organism</th>
<th>X. Laevis</th>
<th>D. Melanogaster</th>
<th>Mouse</th>
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*Homologous γTuRC proteins are in the same row. Numbers refer to the molecular weight of the proteins in kilodaltons.*

Table 2: Components of γTuRCs in different organisms.
Multigene families of γ tubulins

Like α and β tubulin, γ tubulins are encoded by multigene families in some organisms. Initially, Zheng et al. (1991a,b) identified, cloned, and sequenced two γ-tubulin cDNAs from D. melanogaster. Subsequently at least two γ-tubulin genes have been isolated from A. thaliana (Liu et al., 1994), Zea mays (Lopez et al., 1995; Genbank), Euplotes crassus (Tan and Heckman, 1998), Euplotes octocarinatus (Liang and Heckman, 1993), and Paramecium tetraurelia (Ruiz et al., 1999). The identity shared between the two isotypes ranges from 81.6% (D. melanogaster) to 99.5% (Euplotes octocarinatus).

Lajoie-Mazenc and colleagues (1996) cloned a γ-tubulin gene from Physarum polycephalum that is transcribed into a single RNA but the polypeptide seems to be posttranslationally modified into two isoforms of γ tubulin (referred to as a slow and fast form) that have different electrophoretic mobilities, apparent molecular masses (52 kd and 50 kd), and sedimentation properties.

Analysis of the expression patterns of the two divergent D. melanogaster γ-tubulin genes (γTub23C and γTub37CD) reveals that they are differentially expressed. γTub23C is zygotically expressed in males and females during all stages of development. It localizes to centrosomes of mitotic cells and germ cells and the basal bodies of post-meiotic spermatids (Wilson et al., 1997). Mutational analysis of γTub23C reveals that it is required for correct mitotic spindle assembly as well as proper maintenance of the structure of the centrosomes in larvae (Sunkel et al., 1995). The second Drosophila γ-tubulin isotype, γTub37CD, is maternally expressed and localizes to ovaries, and developing egg chambers during
oogenesis, and is utilized by precellular and cellular embryos (Wilson et al., 1997; Tavosanis et al., 1997; Wilson and Borisy, 1998). \( \gamma \)Tub37CD is required for the formation of bipolar mitotic spindles during oogenesis subsequent to germ cell proliferation and during early embryogenesis (Wilson and Borisy, 1998; Llamazares et al., 1999). Wilson and Borisy (1998) have suggested that \( \gamma \)TUB37CD is required for the proper activation of oocytes in meiosis I but is not required for proper meiosis I spindle formation although contradictory data has been presented (Tavosanis et al., 1997; Llamazares et al., 1999).

The two \( \gamma \)-tubulin genes of \textit{A. thaliana} share 98\% aa identity and they are co-expressed in several tissues during different stages of development (Liu et al., 1994). An analysis of the expression patterns of the two \( \gamma \)-tubulin genes of \textit{Euplotes crassus} has not been conducted. However, sequence analysis of the genes reveals that \( \gamma \)tub1 and \( \gamma \)tub2 share 86.5\% aa identity and differ in codon usage, transcriptional initiation sites and poly-A-sites (Tan and Heckman, 1998). \textit{Euplotes octocarinatis} has two isotypes that differ by only two amino acids. Apparently a third \textit{Euplotes} species, \textit{Euplotes aediculatus}, also has two \( \gamma \)-tubulin isotypes that have not been deposited in Genbank as does another ciliate \textit{Blepharisma japonicum} (Tan and Heckman, 1998) indicating that multiple \( \gamma \)-tubulin genes are the norm for ciliates. Since ciliates have multiple types of MTOCs (Adoutte and Fluery, 1996) they may require different isotypes for different MTOCs or the \( \gamma \)-tubulin isotypes may simply fulfill the spatial or temporal regulation of microtubule nucleation required by these organisms. \textit{Z. mays} also has two highly homologous \( \gamma \)-tubulin isotypes; however,
neither their expression patterns nor function has been analyzed (Lopez et al., 1995).

Rationale for my dissertation research

After having previously identified a γ-tubulin gene in humans (which I will call Hγ1) and finding two γ-tubulin genes in D. melanogaster, Zheng (1992) conducted a preliminary search for additional γ-tubulin genes by reprobing a HeLa cDNA library. No additional γ tubulins were found, but subsequent antisense RNA and oligodeoxynucleotide analyses failed to inhibit γ-tubulin function in HeLa cells. This failure of inhibition could have been due to the expression of more than one functionally redundant γ-tubulin gene in HeLa cells. Since multiple γ-tubulin genes were identified in D. melanogaster, another metazoan, and because α and β tubulins exist in multigene families, it is plausible that multiple γ-tubulin genes exist in Homo sapiens.

In recent years the extensive research on the causes and effects of tumorigenesis has revealed that many tumor cells display multiple (supernumerary) centrosomes that nucleate multiple spindles and cause aberrant chromosome segregation leading to genetic instability in these cells. The recent localization of BRCA1, a tumor suppressor gene, to the mitotic centrosome and mutational analysis of this gene has shown that it is involved in the regulation of the G2/M checkpoint and regulation of centrosome duplication (Hsu and White, 1998; Xu et al., 1999). Since γ tubulin has been shown to interact with BRCA1 during mitosis and γ tubulin localizes to supernumerary centrosomes in tumor cells it may be exploited as a target for chemotherapy agents. Bai et al. (1993) also proposed that the cytotoxic antimitotic compound spongistatin 1 may be toxic because of its high affinity for
γ tubulin. Antimitotic agents could also be used to inhibit γ tubulin function and thereby prevent tumorigenesis.

A prerequisite for understanding γ tubulin function and γ tubulin as a target for therapeutic agents in humans is understanding the number and expression patterns of γ-tubulin genes. Since multiple α and β tubulin pseudogenes have been identified in humans, I, like Zheng (1992), chose to probe a Hela cDNA library to search for additional γ-tubulin genes. In chapter 2 of this dissertation I will describe how I used Southern analysis to estimate the total number of γ-tubulin genes found in the human genome. Data is also presented on the identification of a Hγ1 processed pseudogene. Finally, I will also present data on the identification, cloning and sequencing of a second human γ tubulin gene we have named Hγ2. The methods used, results obtained, and a discussion of these results will be presented in Chapter 2.

Since the two γ-tubulin genes are differentially expressed in Drosophila, and I have identified two γ-tubulin genes in Homo sapiens, I have analyzed the expression patterns of these two γ-tubulin genes in various human tissues using RT-PCR analysis. In Chapter 3, I will present a more detailed rationale behind the methodology used and results obtained from the RT-PCR analysis. Since Hγ1 has been mapped to human chromosome 17, I will also describe how Hγ2 was mapped to a specific locus in the human genome.
CHAPTER 2

IDENTIFICATION, CLONING AND ANALYSIS OF ADDITIONAL 𝛾-
TUBULIN SEQUENCES IN HOMO SAPIENS

Introduction

After 𝛾-tubulin genes were identified in Drosophila melanogaster and Homo sapiens (Zheng et al., 1991a), as well as Xenopus laevis and Schizosaccharomyces pombe (Stearns et al., 1991; Horio et al., 1991), it became obvious that 𝛾 tubulin is found not only in Aspergillus nidulans but it is a ubiquitous protein. To date, 𝛾 tubulin has been identified in 36 species and at least two 𝛾-tubulin genes have been identified in six species. Therefore, 𝛾 tubulins, like 𝛼 and 𝛽 tubulins, are encoded by multigene families in some organisms. Three different 𝛾-tubulin cDNAs have been identified in Z. mays (Lopez et al., 1995; Genbank) and Southern blot analysis suggests that as many as five 𝛾-tubulin genes may exist in A. thaliana, however only two have been isolated (Liu et al., 1994). Although Zheng (1992) searched for additional 𝛾-tubulin genes in H. sapiens, none were identified.

Initially Cleveland et al. (1980) used Southern blots of human genomic DNA, probed with chicken 𝛼- or 𝛽-tubulin cDNAs, to estimate that 12-15 𝛼- and 𝛽-tubulin genes each exist in the human genome. Only five 𝛼-tubulin sequences and ten 𝛽-tubulin
sequences have been identified and fully sequenced (see Table 3). Of these fifteen sequences, eight (four α and four β) encode functional genes, while seven (one α and six β) encode pseudogenes (Cowan et al., 1981; Wilde et al., 1982a, b; Lee et al., 1983; Hall and Cowan, 1985). Interestingly, five of the six β-tubulin pseudogenes are processed pseudogenes and only one is a "classical" pseudogene (see Table 3). Processed pseudogenes are inactive genomic sequences that resemble the mRNA transcript of a functional gene in that they lack introns and end with a poly(A)tail. Processed pseudogenes are often flanked by short direct repeats believed to have originated from the reverse transcription of the mRNA of a functional gene followed by a transposition event in which the transcript is inserted at some random site in the genome (reviewed in Lewin, 1997). In order for processed pseudogenes to be propagated in the human genome, these sequences must be integrated in germline cells (reviewed in Lewis and Cowan, 1986). Classical pseudogenes have the same structure as functional genes, but they have been inactivated by mutations that prevent the proper transcription or translation of the gene (reviewed in Lewin, 1997). These pseudogenes are usually found adjacent to a functional gene since many of them appear to have resulted from duplications of genomic sequences. To date no γ-tubulin pseudogenes have been identified in higher eukaryotes.

Several of the human α- and β-tubulin genes have been mapped to chromosomes (see Table 3). The human β-tubulin gene TUBB (Tubulin B, also designated M40 protein) has been localized to the short arm of chromosome 6 at 6p21.3 using
<table>
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<td>Pseudo-Bα1</td>
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*Expressed-functional gene; Pseudo-classical pseudogene; Processed-processed pseudogene

**Only first authors are listed in the reference column.

Table 3: Human α- and β-tubulin gene chart.
human/chinese hamster ovary somatic cell panels (Floyd-Smith, 1986). Two processed pseudogenes of TUBB, designated TUBBP1 (previously identified as 21β) and TUBBP2, localize to chromosome 8q region 21, and chromosome 13, respectively (Floyd-Smith, 1987). The fact that TUBB and its two pseudogenes are dispersed in the human genome is characteristic of processed pseudogenes. Other examples of dispersed processed pseudogenes have been documented in several multigene families such as the globin gene family (Fritsch et al., 1980; Leder et al., 1981). Two of the other three functional β-tubulin genes TUBB4 and TUBB5 (5β) have been localized to human chromosomes 16 and 19 respectively by various labs involved in the human genome project (www.ncbi.nlm.nih.gov-select Human genome resources). TUBB2 (Hβ2) as well as the other β-tubulin pseudogenes listed in Table 3 have not been mapped to chromosomes.

The first α-tubulin gene identified in humans, Kα1, (K-alpha-1; Cowan et al., 1983) has not been localized to a chromosome. In an attempt to identify candidate genes involved in two types of human nonsyndromic deafness, Dode et al. (1996) localized TUBA2 (Tubulin alpha-2), another α-tubulin gene, to region q11 of chromosome 13. However, mutations in TUBA2 are now known not to be the cause of nonsyndromic deafness. TUBA1 (Hα2) and TUBA3 (bα1) have been localized to the q arm of chromosome 2 by labs involved in the human genome project (www.ncbi.nlm.gov). Although both of these genes localize to 2q, their position on chromosome 2 in relationship to each other has not been established.

In most of the organisms in which multiple tubulin genes have been identified, they
are dispersed throughout their respective genomes. However, α- and β-tubulin genes are arranged in clusters or tandem repeats in some unicellular parasites, sea urchins, antarctic fish, and red algae (Alexandraki and Ruderman, 1983; Parker and Dietrich, 1997; Akkari, 1997). For example, in the parasitic protozoan, *Leishmania*, α- and β-tubulin genes are arranged in separate tandem repeats, whereas α- and β-tubulin genes in *Trypanosoma brucei* are arranged in alternating tandem repeats (Landfear *et al.*, 1983; Thomashow *et al.*, 1983). It has been suggested that these genes are arranged in tandem as a means to regulate gene expression through common DNA regulatory elements (reviewed in Cleveland and Sullivan, 1985). Of the six species in which multiple γ-tubulin genes have been identified, only the two γ-tubulin genes isolated from *D. melanogaster* (γTUB23C and γTUB37CD) have been mapped to chromosomes and, as is denoted by the numbers in the names of the genes, they are not arranged in tandem (Zheng, 1992; Wilson *et al.*, 1997).

Since multiple γ-tubulin genes have been identified in *D. melanogaster*, a metazoan, as well as five other species of plants and animals and because α and β tubulins are encoded by multigene families, it is plausible that multiple γ-tubulin genes exist in *Homo sapiens*. I have, therefore, attempted to estimate the number of γ-tubulin genes in the human genome using Southern analysis of human genomic DNA. From this analysis I have estimated that no more than three γ-tubulin sequences exist in the human genome.

I have chosen to screen a HeLa cDNA library to search for additional γ-tubulin genes. I have isolated cDNAs that are encoded by a novel γ-tubulin gene which I have named Hγ2. I have sequenced these cDNAs as well as a full-length cDNA product of this
gene identified initially as an Expressed Sequence Tag (EST) clone. Hy2 is highly homologous to Hγ1 and encodes a functional (expressed) gene. I have also sequenced a genomic γ-tubulin clone we obtained from Dr. Helmut Shraudolf’s lab (Universität Ulm, Ulm, Germany) that I named HyF for Human gamma fragment. Characterization of HyF using a BLASTN search has revealed that this fragment originated from a processed pseudogene, located on human chromosome 7, that probably is derived from Hγ1.

Although the Hγ1 gene has been identified in the form of a cDNA clone, a genomic Hγ1 clone has not been identified. In the early 1990's while several labs were trying to identify a locus which contains the gene that causes familial breast cancer, the Hγ1 genomic locus was mapped to within ~150 kb centromeric to the BRCA1 (breast and ovarian cancer 1) susceptibility gene on region q21 of chromosome 17 (Miki et al., 1994; Friedman et al., 1995; Rommens et al., 1995). All the labs involved in the search for BRCA1 have used YAC clones that contain the 1 Mb BRCA1 region of chromosome 17 to probe cDNA libraries in order to identify any functional genes, such as Hγ1, in the region (Brody et al., 1995; Couch et al., 1995; Friedman et al., 1995; Rommens et al., 1995).

In collaboration with Dr. Ralf Krahe’s Lab (Dept. of Human Cancer Genetics, OSU) we have determined the localization of Hy2 in the human genome. This mapping analysis has revealed that Hy2, like Hγ1, is located on chromosome 17, between 17q12 and 17q21. Further investigation of the location of Hγ1 in relation to Hy2 using PCR analysis has revealed that the Hy2 gene is located on the same 700 kb YAC as the Hγ1 gene. To date we have not determined if these genes are arranged in tandem from the
experiments we have performed.

Materials and Methods

Isolation of HeLa genomic DNA

HeLa cells were kindly provided by Dan Luo (Dept. of Molecular Genetics, OSU). Genomic DNA was isolated from HeLa cells as described by the "Isolation of DNA from Mammalian Cells: Protocol I" in Sambrook et al. (1989). The absorbance of the DNA was measured at 260 nm and 280 nm in order to determine the concentration and purity of the DNA and then it was visually analyzed on 0.7% agarose/TBE gels [AgaroseLE, Boehringer Mannheim Biochemicals (BMB); TBE is 5.4 g/l of Tris [hydroxymethyl]amino-methane, 2.7 g/l boric acid and 0.465 g/l EDTA).

Southern Analysis

Ten micrograms of DNA were digested with the appropriate restriction endonuclease as directed by the manufacturer’s protocol (New England Biolabs). Digested DNA was concentrated in Microcon 30 filter cups (Amicon, Inc.) and run on 0.7% agarose/TBE gels (AgaroseLE, BMB). The gels were stained with 1 µg/ml of ethidium bromide (Sigma), photographed, and denatured in 50 mM NaOH for 45 min at room temperature. They were rinsed three times in dH₂O and soaked for 30 min in dH₂O. They were then dried onto Whatman 3MM paper (10 min heat off, 15 min at 65°C, 15 min heat off). The dried gels were floated off 3MM paper in dH₂O and prehybridized in BLOTTO
(Bovine Lacto Transfer Technique Optimizer, Johnson et al., 1984) for 1 hour at 65°C or 60°C. After prehybridization, probes were added to 1x10^7 cpm/ml of BLOTTO and hybridizations were carried out at either 65°C or 60°C for 20-24 hours. Gels probed at 65°C were washed twice in 2x SSC (0.6 M NaCl, 34 mM sodium citrate, pH=8), 0.1% SDS, for 20 min/wash and twice in 0.2x SSC, 0.1% SDS, also 20 min/wash. All washes were at 65°C. Gels hybridized at 60°C were washed three times at 60°C for 10 min with 2x SSC, 0.1% SDS. A Molecular Dynamics phosphor screen or Kodak X-OMAT AR film was used for autoradiography.

Probes were made from 5' and 3' regions of a Hyl cDNA. Two 3' probes were produced as follows. Plasmid pH3 (a pBluescript sk+(Stratagene) based plasmid containing the Hyl cDNA in an EcoRI site) was digested with PstI and ClaI to produce a 399 bp fragment corresponding to aa298 to 432 or with BamHI to produce a 528 bp fragment corresponding to aa339 to 451 plus 191 bp of the 3'UTR. These fragments were recovered from agarose gels by the Freeze-Squeeze method (Tautz et al., 1983) and were radiolabeled by the method of Feinberg and Vogelstein (1983) using random nine-mers (Stratagene). A 515 bp 5' probe, that begins 24 bp 5' to the start codon and ends at aa179, was generated by radioactive PCR. Thirty cycles of a first round PCR reaction of 100 μl were carried out with 10 ng of plasmid pH3 DNA, 0.1 pmoles each of M13S-20 universal primer and HGTR2 (a Hyl reverse primer: cactgagtatgtct made by Operon Technologies), 0.2 mM each of dATP, dCTP, dGTP, dTTP (BMB), and 2.5 units of ID-Pol DNA polymerase (ID Labs, Inc.) in 1x NH₄⁺ ID-Pol PCR buffer (16 mM (NH₄)₂SO₄, 67 mM...
Tris-HCl (pH=8.8 at 25°C), 0.1% Tween-20} with MgCl₂ added to a final concentration of 0.75 mM. Five percent (5 μl) of the first round product was used as a template for a radioactive, 25 cycle, second round PCR amplification using 0.6 μM [α³²P]-dATP (ICN) and 0.2 mM each of cold dCTP, dGTP, and dTTP (BMB). Amplification conditions for both rounds of PCR were 1 min at 94°C and 4 min at 50°C following an initial 2 min, 94°C hold. Excess primers and nucleotides were separated from all probes using Microcon-30 microconcentrators (Amicon, Inc.).

**Sequence analysis of a genomic human γ-tubulin clone (HyF)**

A 930 bp human genomic DNA fragment of a putative second γ-tubulin gene, in plasmid pIBI (IBI-Eastman Kodak) was received from Dr. Helmut Schraudolf's lab (Universität Ulm, Ulm, Germany). The 930 bp insert was subcloned into an EcoRI/BamHI site of M13mp18 and M13mp19. I named this new fragment HyF for human gamma fragment. Following single-stranded DNA preparations (Sambrook *et al.*, 1989) of M13mp18 and M13mp19 containing the insert DNA, I sequenced the clones using the Sequenase 2.0 (USB-Amersham Life Sciences) standard protocol.

**Library screening**

A HeLa λZapII cDNA library (Stratagene) was screened using the plaque lift and purification protocol described by Carlock (1988) with the following modification. The filters were neutralized by laying them plaque side of the filter up, on 3MM paper soaked in
3M NaCl, 0.5 M Tris-HCl, pH=7.0 for 10 min. After the filters were air-dried the plaque DNA was cross-linked to the filters using a UV Stratalinker 1800 (Stratagene). Filters were prehybridized in BLOTTO for four hours and hybridized to an [$\alpha^{32}$P] dATP random-labeled (Feinberg and Vogelstein, 1983) 930 bp, HγF probe, for 12-18 hours at 65°C. Filters were washed twice in 2x SSC, 0.1% SDS at 65°C followed by two washes in 0.2x SSC, 0.1% SDS for 25 minutes/wash. From a total of 2.5 x10^6 plaques screened, 21 positive clones were isolated.

$\lambda$ZapII phage have been designed to allow the isolation of the Bluescript plasmid containing an insert from the phage using helper phage R408 (Stratagene). Clones were excised from positive plaques and recircularized using helper phage by following the $\lambda$ZapII protocol provided by Stratagene. Rescued pBSsk- plasmids containing the inserts of interest were prepared using the standard large scale plasmid preparation protocol found in Sambrook et al. (1989), except that the volume of the prep and components used in the prep were reduced to 1/5. Plasmids were purified by two rounds of CsCl-ethidium bromide equilibrium density centrifugation.

All 21 clones were sequenced at both ends using Sequenase 2.0 (USB-Amersham Life Sciences) to determine if their sequences matched the sequences of Hγ1 or HγF, or were novel. Four novel clones (pDOW1a, pDOW7, pDOW12, and pDOW21) were obtained and were sequenced on both strands by primer walking using the Sequenase 2.0 (USB-Amersham Life Sciences) protocol.
EST database search, subcloning and sequencing of a full-length Hγ2 clone

In order to obtain a full-length Hγ2 clone, the human expressed sequence tags (EST) database (www.ncbi.nlm.nih.gov/dbEST) was searched and 159 EST sequences were analyzed. A full-length Hγ2 clone, that I named Hγ2EST, was found. This clone was purchased from Research Genetics, Inc. and was sequenced fully on both strands. Most sequencing was carried out using the double-stranded sequencing protocol for Sequenase 2.0 (USB-Amersham Life Sciences). A portion of one strand was sequenced using the ABI Prism Dye Terminator Cycle sequencing protocol (Perkin Elmer). Cycle sequencing reactions were run on a GeneAmp PCR 2400 thermal cycler (Perkin Elmer) and sequences were read with an ABI 373A DNA Sequencer (Perkin Elmer). Basic sequence analysis was done using DNA Strider 1.2 (Marck, 1988). Alignments of the γ-tubulin genes were carried out with the ClustalW algorithm (Thompson et al., 1994) and SeqVu software (Garvan Institute, Sydney, Australia).

Construction of plasmid pASHγ2

Five nanograms of 2x CsCl purified-plasmid pHγ2EST [the Hγ2EST clone in plasmid Lafmid BA (ATCC)] DNA was used as a template to amplify a 1589 bp region of the plasmid that includes the entire coding region and 205 bp of the 3’ UTR. Thirty cycles of amplification using 2.5 units of Taq DNA polymerase (Gibco BRL) were carried out using the components and amounts recommended in the Gibco BRL protocol. Hγ2EST specific primers (HGEST3F1: gaagatcgagcgtgccgag; and HGEST3R1:
gaagatctgacaaccaagctttat) with BglII ends were used to amplify pHγ2EST using the following thermocycling parameters: 94°C for 2 min; 30 cycles of 94°C for 1 min and 60°C for 4 min. Taq DNA polymerase was removed from the PCR reaction using Strataclean Resin (Stratagene) and the sample was concentrated in a Microcon-30 microconcentrator (Amicon, Inc.). This sample was digested with BglIII as directed by the manufacturer (New England Biolabs) and then purified from a 0.7% SeaplaqueGTG low melting point agarose gel (FMC) using Wizard PCR preparations (Promega Life Sciences).

Plasmid pAS248 (Toda et al., 1991) was digested with BamHI as directed by the manufacturer (New England Biolabs). The BamHI enzyme was removed from the sample using Strataclean Resin (Stratagene) and the sample was concentrated in Microcon-30 microconcentrators (Amicon, Inc.). The digested sample was treated with 0.01 units of Thermosensitive Alkaline Phosphatase (TsAP) (Gibco BRL) in TsAP buffer supplemented with MgCl₂ to a final concentration of 3mM at 65°C for 20 min and then inactivated following the manufacturer’s protocol.

The 1589 bp, BglII-digested Hyγ2 clone was ligated [with T4 ligase (BMB)] into the BamHI site of pAS248, which is located in a small polycloning site immediately downstream of the S. pombe alcohol dehydrogenase promoter (Russell and Hall, 1983). The ligation mix was used to transform CaCl₂ competent JM109 (E. coli) cells (Sambrook et al., 1989) and Wizard DNA minipreps were performed using the manufacturer's protocol (Promega Life Sciences). Test digests were performed on the miniprep DNAs to determine if the ligation was successful. A correct plasmid was identified and named
pASHγ2. A standard large scale plasmid preparation (Sambrook et al., 1989) was performed and the plasmid was purified by two rounds of equilibrium centrifugation in CsCl-EtBr gradients. Purified pASHγ2 was sent to Dr. Tetsuya Horio for transformation into *S. pombe* strains.

**Chromosomal mapping**

Primer combinations were designed to give specific amplification of Hγ2 and were used in PCR reactions to map Hγ2. A forward primer, HUGAM1-3F1: aggacaactttgatgagatggaca, with a sequence common to the coding regions of Hγ1 and Hγ2 was manufactured by Operon Technologies. Two reverse primers corresponding to sequences in the 3'UTR of Hγ2 and specific for Hγ2, [HUGAM3R1: ctggagatgaacgaagaaggttg, and HUGAM3R2: atctgaaggagaaggtagtg], were manufactured by Operon Technologies. HUGAM1-3F1 and HUGAM3R1 should amplify a band of 252 bp specifically from Hγ2, while HUGAM1-3F1 and HUGAM3R2 should amplify a 151 bp band specifically from Hγ2. Because the hybrid panels used in chromosomal mapping are composed of human genomic DNA and Chinese hamster ovary (CHO) DNA, it was important to determine if these primer combinations amplified bands from CHO genomic DNA. Therefore, CHO genomic DNA and human genomic DNA were tested for specificity of the primers for human genomic DNA. Thirty cycles of first round PCR reactions of 100 µl each were carried out with 100-200 ng of CHO genomic DNA or human genomic DNA, 0.2 pmoles each of HUGAM1-3F1 and HUGAM3R1 or
HUGAM3R2, dNTPs at 0.2 mM each, and 2.5 units of ID-Pol DNA polymerase (ID Labs, Inc.) in 1X NH$_4$+ID-POL PCR buffer [16mM (NH$_4$)$_2$SO$_4$, 67mM Tris-HCl (pH=8.8 at 25°C), 0.1% Tween-20] supplemented with 0.50 mM MgCl$_2$ (final concentration). The specificity of each of the aforementioned primer combinations for amplification of Hy2 versus Hy1 was also tested prior to chromosomal mapping as stated in the Materials and Methods of Chapter 3.

HUGAM1-3F1, HUGAM3R1, and HUGAM3R2 were given to Dr. Ralph Krahe's lab in the Dept. of Human Cancer Genetics at the Ohio State University. They used two independent human-CHO cell hybrid clone panels to map Hy2 to a chromosome. Panel 1 was a multi-chromosomal 17-member human-CHO cell hybrid clone panel (Stallings et al., 1988). At least two independent PCRs were carried out on Panel 1 using each set of primers. The PCR parameters for HUGAM1-3F1 and HUGAM3R1 were 94°C for 5 min, followed by 35 cycles at 94°C for 30 sec, 67°C for 30 sec, and 72°C for 30 sec, and a final extension for 10 min at 72°C. The parameters were the same for the HUGAM1-3F1 and HUGAM3R2 combination except that the annealing temperature during the 35 cycles of amplification was at 55°C. PCR products were analyzed by standard 1.5% agarose gel electrophoresis. Human and CHO genomic DNAs were used as positive and negative controls for amplification with each PCR reaction. Discordancy analysis was calculated for each chromosome using Lotus 123.

The second panel (Panel 2) was a mono-chromosomal human-CHO cell hybrid clone panel and was used to verify the mapping results obtained using the multi-
chromosomal panel. The same PCR reaction parameters as those used on the multi-
chromosomal panel were used to amplify Hy2 DNA from this panel.

In order to localize Hy2 to a more specific region of a human chromosome, the 93-
member Whitehead Institute Genebridge 4 radiation hybrid (GB4RH) panel was used.
PCR analysis was carried out using the same parameters used on Panel 1 and Panel 2.
PCR reactions were resolved on 6% denaturing polyacrylamide gel electrophoresis
followed by silver staining. They were then scored to generate a linear vector of numbers
that reflect the presence or absence of positive PCR amplification (reviewed in Miano et al.,
1999) and the results were submitted to the Whitehead Institute/MIT center for Genome
Research Radiation Hybrid mapping server (http://www.genome.wi.mit.edu). The map
positions and distances of STS markers are expressed in centiRays (cR) where 1 cR equals
~ 300 kb for the GB4RH panel. The map position of Hy2 was determined relative to an
STS marker and its distance was estimated in cR.

Preparation of YAC DNA for PCR analysis

We received a yeast strain from Dr. Johanna Rommen’s lab (University of Toronto)
containing yeast artificial chromosome (YAC) HSD2 subclone 'C' (Rommens et al., 1995)
which maps to the HSD17B locus on the long arm of chromosome 17 (17q21) and carries
the Hyl gene. This strain was grown on YPD (2% w/v peptone, 1% w/v yeast extract, 2%
glucose, 1.8% w/v agar) plates at 30°C. Low molecular weight YAC DNA was prepared
by modifications of the protocols of Carpten (1994) and Kaiser et al. (1994). Five milliliter
cultures inoculated with single colonies from YPD plates were grown in 25 ml erlenmeyer flasks to saturation, (30°C for 36 hours) in YPD medium with shaking at ~200 rpm. Cells were pelleted at 1000Xg and rinsed with 100 µl of 50 mM EDTA, pH=8.0. Pellets were resuspended in 500 µl of SPEM buffer (1M sorbitol, 100 mM sodium phosphate, pH=7.4, 60 mM EDTA, pH=8.0, and 90 mM β-mercaptoethanol). Zymolyase 100T (Seikagaku KOGYO, Co., LTD., Tokyo, Japan) was added to a concentration of 0.5 mg/ml and cells were incubated at 37°C for 1-2 hours to give 90-100% spheroplasting. Cells were examined for spheroplasting using phase contrast light microscopy after treating 2.5 µl of cells with 10 µl of 5% SDS. Spheroplasts were harvested by centrifugation at 600Xg and resuspended in 500 µl of yeast lysis buffer (0.5 M NaCl, 50 mM Tris-Cl, pH=8.0, 25 mM EDTA, pH=8.0, 30 mM β-mercaptoethanol, 1% w/v SDS, 0.1% v/v NP-40). Cells were lysed by incubation at 68°C for 15 minutes and transferred to 2 ml microcentrifuge tubes. 200 µl of 5M potassium acetate was added to the samples and they were incubated on ice for 1 hour. After a 5 minute centrifugation in a microcentrifuge the supernatant was transferred to a fresh microcentrifuge tube and precipitated with 1 volume of 100% isopropanol at room temperature. The DNA was pelleted by centrifugation and the pellet was air-dried and resuspended in 100 µl of TE, pH=7.4. RnaseA (Dnase-free) (Sigma) was added to a final concentration of 1 mg/ml and the sample was incubated at 37°C for 30 minutes. One-tenth volume of 3M sodium acetate, pH=5.2, was added to the sample and it was precipitated with 200 µl of 100% isopropanol. The pellet was air-dried then resuspended in 100 µl of TE, pH=7.4. The concentration of the YAC DNA was
determined by measuring its absorbance at 260 nm.

YAC DNA from HSD2 subclone 'C' was given to Dr. Ralf Krahe's lab (Dept. of Human Cancer Genetics, OSU). They used the same primer combinations (HUGAM1-3F1 in combination with HUGAM3R1 or HUGAM3R2) and PCR conditions that they used in the chromosomal mapping experiments in the previous section in order to determine if the Hγ2 gene maps to YAC-HSD2. PCR products were run on a 1.5% agarose gel and visualized using EtBr.

**PCR analysis and DNA sequencing of HγF**

Two specific primers, HGENO1B: gcggatcgcctcaagggcacca and HGENO2E: ggaattcggtccatagtcagct, were designed from the sequence of the 930 bp clone, to amplify a 761 bp fragment from human genomic DNA. These primers were manufactured by Operon Technologies. HeLa cells were obtained from Dr. James Lang's lab (Dept. of Internal Medicine, OSU) and genomic DNA was isolated as is described in Sambrook *et al.* (1989). Human DNA, obtained from a patient at the OSU Hospital, was given to us by Dr. Arthur Burghes' Lab (Dept. of Neurology, OSU). HGENO1B and HGENO2E were used to amplify the insert from this HeLa genomic DNA and human patient genomic DNA. A second specific reverse primer, HGENOR2: gtgcaggatctggct, was designed and used with HGENO1B to amplify a smaller 576 bp region of HγF from human genomic DNA. Platinum Taq DNA polymerase (2.5 units/100 μl reaction) (Gibco BRL) was used to amplify 500 ng of human genomic DNA using the manufacturer's suggested PCR conditions.
solutions. The thermocycling parameters for both sets of primers were 94°C for 2 min, followed by 30 cycles at 94°C for 1 min and 55°C for 4 min. Ten percent (10 µl) of the first round PCR product was used as a template for 25 cycles of second round amplification. Second round PCR products were run on a 1% SeaplaqueGTG low melting point agarose gel (FMC) and were purified using the Wizard PCR prep system (Promega Life Sciences). Purified samples of the 576 bp fragment of human genomic DNA were sent to the University of Georgia’s Molecular Instrumentation Facility (Athens, Ga; www.mgif.uga.edu) for sequence analysis. Basic DNA analyses were carried out using DNA Strider 1.2 software (Marck, 1988). Alignments of the sequence were done with the ClustalW Algorithm (Thompson et al., 1994) and SeqVu software (Garvan Institute, Sydney, Australia).

**Blast search for HγF**

In order to find a full-length clone containing HγF, the Hγl sequence and the 930 bp HγF sequence were used to search the Washington University (St. Louis) human genomic DNA database (http://genome.wustl.edu/gsc) using the default parameters and selecting the GSS—genomic sequence survey database of the BLASTN program (Altschul et al., 1990). Sequence alignments were carried out using ClustalW (Thompson et al., 1994) and SeqVu (Garvan Institute, Sydney, Australia) to compare the clones to Hγl and HγF.
Results

Estimation of the number of γ-tubulin sequences in *Homo sapiens*

In order to estimate the number of γ-tubulin sequences in *Homo sapiens*, I performed Southern hybridizations on HeLa genomic DNA digested with four different restriction enzymes (EcoRI, HindIII, BamHI, and SacI). The hybridizations were carried out at 60°C or 65°C under the salt conditions given in the Materials and Methods. In 60°C hybridizations, the 5' probe revealed two or three bands of hybridization in each restriction enzyme digest (Figure 2). This probe also hybridized faintly to a 4.8 kb band in the *A. nidulans* lane, that is the expected size of an EcoRI fragment that contains the *A. nidulans* γ-tubulin gene. This probe shares 54.2% nucleotide identity with the corresponding *A. nidulans* sequence. Since this probe detected an *A. nidulans* γ-tubulin sequence at 60°C, we can conclude that any human γ-tubulin not detected under our conditions would have to be extremely divergent from Hγ1. The 3' BamHI probe also showed two or three bands of hybridization (Figure 3) in each digest at 60°C but did not show a band of hybridization in the *A. nidulans* DNA. The 3' probe shares only 32.1% with the corresponding *A. nidulans* γ-tubulin sequence and, therefore, may be too divergent to hybridize with *A. nidulans* DNA under the conditions used. Hybridizations carried out at 65°C using either the 5' probe or the 3' (PstI/ClaI) probe showed similar results except that neither probe showed hybridization to *A. nidulans* DNA (data not shown). From these data we can conclude that no more than three γ-tubulin sequences are detected under these conditions.
and therefore there are probably no more than three \( \gamma \)-tubulin sequences in the human genome.

**Identification and characterization of a novel human \( \gamma \)-tubulin cDNA, \( \text{Hy}2 \)**

A 930 bp human \( \gamma \)-tubulin fragment was obtained by PCR amplification of human genomic DNA and was provided to us by Dr. Helmut Schraudolf (Universität Ulm). I subcloned this fragment into M13mp18 and M13mp19 to facilitate single-stranded dideoxy sequencing. Sequencing analysis of this fragment which I named \( \text{HyF} \) (Figure 4) revealed that it shares 91.6% nucleotide identity and 86.1% amino acid identity with the corresponding region of \( \text{Hy}1 \) (Figure 5). These results indicated that \( \text{HyF} \) is a novel sequence and it had not been amplified from \( \text{Hy}1 \). The 930 bp/310 aa \( \text{HyF} \) clone spans more than two-thirds of the \( \text{Hy}1 \) cDNA sequence (see Figure 5) and, interestingly, it does not contain any intron sequences.

In an effort to clone a cDNA corresponding to \( \text{HyF} \), I used the \( \text{HyF} \) fragment as a probe to screen a HeLa \( \lambda \) ZapII cDNA library at high stringency. I screened 2.5 \( \times \) 106 plaques over four rounds of purification and isolated 21 positive clones. Partial sequencing of the clones revealed that none of them corresponded to \( \text{HyF} \). Fifteen of the clones corresponded to \( \text{Hy}1 \), one clone contained a human \( \alpha \)-tubulin sequence, and four of the clones, (pDOW1a, pDOW12, pDOW7, pDOW21) represented a novel human \( \gamma \)-tubulin
Figure 2: Low stringency Southern analysis of human genomic DNA using a 5'-H\gamma\lambda probe.

Hela genomic DNA samples (10 \mu g each) were digested with EcoRI, HindIII, BamHI, or SacI. *A. nidulans* genomic DNA (labeled AspE) was digested with EcoRI in order to serve as a control for hybridization stringency. Digests were run on 0.7% agarose gels, processed for Southern analysis, and probed with a 515 bp 5'-H\gamma\lambda probe at 60\degree C. The positions of the \lambda HindIII molecular weight marker bands are shown to the right of the gel.
Figure 2.
Figure 3: Low stringency Southern analysis of human genomic DNA using a 3'-Hγ1 probe.

HeLa genomic DNA samples (10 μg each) were digested with EcoRI, HindIII, BamHI, or SacI. Digests were run on 0.7% agarose gels, processed for Southern analysis, and probed with a 528 bp 3'-Hγ1 sequence at 600C.
Figure 3.
Figure 4: The nucleotide sequence of HγF and its predicted amino acid sequence.

The HγF genomic clone we received from Dr. Helmut Shraudolf and sequenced on both strands. This clone is 930 base pairs long, encodes a 310 amino acid protein, and lacks introns.
Figure 4.
cDNA that I have named Hγ2 (Human gamma 2). None of these four clones, however, encoded a full-length Hγ2 sequence. Two clones (pDOW7 and pDOW21) (Figure 6) contained 1465 bp inserts with identical sequences and two clones (pDOW1a and pDOW12) (Figure 7) contained 809 bp inserts that are identical to each other and to the corresponding regions within the larger inserts. The two larger clones lacked the region corresponding to the N-terminal 48 amino acids of Hγ1 (see Figure 8) but extended into the 3' UTR region which contains a poly(A) signal (AATAAA) and ended in a poly(A) tail.

In order to identify a full-length Hγ2 clone, I searched the human Expressed Sequence Tag database (dbEST) maintained by the National Center for Biotechnology Information (NCBI). Thirty-nine of 159 EST sequences analyzed encoded partial Hγ2 sequences (the other ESTs encoded Hγ1). A full-length cDNA clone (Accession No H10668 and H10669) obtained from a human infant (73 days postnatal) brain cDNA library was identified as corresponding to Hγ2 based on the EST sequence which covered approximately 450 bp at each end of the clone. This cDNA clone (which I named Hγ2EST) was obtained from Research Genetics and sequenced on both strands. The cDNA is 1857 bp in length and contains the entire Hγ2 coding region as well as 244 bp of the 5'UTR and 232 bp of the 3' UTR (Figure 9). The sequence of Hγ2EST was identical to the corresponding regions of the cDNA in pDOW7 and pDOW21, except that Hγ2EST contained a 27 bp in-frame insert within the coding region that was not present in the corresponding region of pDOW7 and pDOW21, nor the same region in pDOW1a and
Figure 5: A comparison of the predicted amino acid sequences of HγF and Hγ1.

Abbreviations: H.g1-predicted amino acid sequence of Hγ1; H.gF-predicted amino acid sequence of HγF.

The HγF clone spans more than two-thirds of the Hγ1 cDNA sequence and shares 86.1% aa identity with the corresponding region of the Hγ1 cDNA.
Figure 5.
Figure 6: The nucleotide and predicted amino acid sequence of clones pDOW7 and pDOW21.

The sequence shown in this figure corresponds to the 1465 bp sequence of two identical clones, pDOW7 and pDOW21, that were identified by screening a HeLa cDNA library with HyF. The sequence of these clones spans 89.4% of the coding region of a novel γ-tubulin gene, Hy2. The predicted amino acid sequence of the novel γ-tubulin gene contains 404 amino acids in the coding region of the gene. The stop codon is underlined in the figure. This sequence contains a polyadenylation signal sequence (underlined) and it ends at the poly(A)tail.
(To be continued)
(Figure 6 continued)

1021/341
tcc tgc tcc ttt gaa agt tcc tgc cag cag ttt gac aag ctg cgg aag cgg gat gcc ttc ser ser leu phe glu ser ser cys gln gln phe asp lys leu arg lys arg asp ala phe 1081/361
ttc gag cag ttc cgt aag gag gac atg ttc aag gag aac ttt gat gag atg gac agg tct leu glu gln phe arg lys glu asp met phe lys asp asn phe asp glu met asp arg ser 1141/381
agg gag gtt gtt cag gac ctc att gat gag tac cat ggc gcc acc cag cca gac tac att arg glu val val gln glu leu ile asp glu tyr his ala ala thr gln pro asp tyr ile 1201/401
tcc tgg ggc acc cag gag cag tga ttcccctccccactactctctctctctctctagatggtaaccacagcctc ser trp gly thr gln glu gln OPA 1275

gaccatgcttgtctctcctgacccccttcacctaatgtgcttcatctctcagccccgctgtgtgtctctgctctctctctctccattgctacttttaatagtgtgttctagctctactaatttatttttatattttttatatattttttagtttagtttagttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttagtttag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Figure 7: The nucleotide and predicted amino acid sequence of clones pDOW1a and pDOW12.

The nucleotide sequence shown on this figure corresponds to the 809 bp sequences of two identical cDNA clones, pDOW1a and pDOW12, that were identified by screening a HeLa cDNA library with HγF. The predicted amino acid sequence contains 269 amino acids. These clones encode a portion (59%) of a novel γ-tubulin gene, Hγ2. This sequence is contained within the sequence of clones pDOW7 and pDOW21.
gac atc ttt gac atc ata gac cga gaa gca gat gga aqt gag ggc ttc gtg
asp ile phe asp ile ile asp arg glu ala asp gly ser asp ser leu glu gly phe val
61/21
c tg tgt cac tcc atc gct ggg gtt aag ggt tct ggc gtc tcc tac ttc tct gag cga
leu cys his ser ile ala gly gly thr gly ser gly leu gly ser tyr leu leu glu arg
121/41
c tg aat gac agg tac ccc aag aag cta gtg cag act tat tca gtg ttt ccc tac cag gac
leu asn asp arg tyr pro lys lys leu val gin thr tyr ser val phe pro tyr gin asp
181/61
gag atg agc gac gta gtg gtt cag ccc tac aat tca ttc atc cta ctc cag aag cgg att gcc aca gac
glu met ser asp val val val val gin pro tyr asn ser leu leu thr leu lys arg leu thr
241/81
cag aac gca gat tgt gtg gtg cag ac aca ggc ctt aac cgg att gcc aca gac
glu asn ala asp cys val val leu asp asn thr ala leu asn arg ile ala thr asp
301/101
c gc ctc cac atc cag aac cgg tcc ttc tcc cag atc aac cag ctt ggt tcc acc atc atg
arg leu his ile gin asn pro ser phe ser gin ile asn gin leu val ser thr ile met
361/121
tgc gcc agc acc acc acc ctt cgc tac ccc ggc tac atg aac aat gac ctc atc gcc ctc
ser ala ser thr thr thr leu arg tyr pro gly tyr met asn asn asp leu ile gly leu
421/141
tgc gtc tcc atc att ccc acc cca cgg ctc cac ttt ctc atg acc gcc tac acc ccg ctc
ile ala ser ile pro thr pro gin pro arg pro leu his phe leu met thr gly tyr thr pro leu
481/161
c gc gac cag cag tca gtg gcc agc gta ggg aag aac acc cgc gta gtg gtt ctc atg agg cgg
thr thr asp gin ser val ala ser val arg lys thr thr val leu asp val met arg arg
541/181
c tg ctc cag ccc aag aac gtg atg gtg tcc aca ggc cga gac cgc cag acc aac cag tgc
leu leu gin pro lys asn val met val ser thr gly arg asp arg gin thr asn his cys
601/201
tac atc gcc atc tcc acc atc atc cag gga gag gtt gcc ccc acc cag gtc cac aag agc
tyr ile ala ile leu asn ile ile gin gly gly glu asp pro thr gin val his lys ser
661/221
c tg cag cag atc ccc aac cgg gag cgg aag tgg gcc acc atc atc ccc gtc ggg gcc gcc agc atc
leu gin arg ile arg gin arg lys leu ala asn phe ile pro trp gly pro ala ser ile
721/241
cag cgg gtc cgg tgg gtt gcc ccc cac cgg gtc agc ggg ctc
gln val ala leu ser arg lys ser pro tyr leu pro ser ala his arg val ser gly leu
781/261
atg atg gcc aac cac acc agc atc tcc
met met ala asn his thr ser ile ser

Figure 7.
Figure 8: A comparison of the predicted amino acid sequences of \( \text{Hy}_2 \)-clone pDOW7 and \( \text{Hy}_1 \).

Abbreviations: \text{Hgam1}-predicted amino acid sequence of \( \text{Hy}_1 \); \text{Hy}_2/\text{pD7}-predicted amino acid sequence of the \( \text{Hy}_2 \) insert in pDOW7.

The predicted amino acid sequence of the pDOW7, partial \( \text{Hy}_2 \) insert is highly homologous to \( \text{Hy}_1 \). The clone lacks the region corresponding to the N-terminal 48 amino acids of \( \text{Hy}_1 \). Amino acid differences are indicated by asterisks.
<table>
<thead>
<tr>
<th>hgam1</th>
<th>MPREIITLQL GQCNGQIGFE FWKQLCAEBG ISPEAIVVNEF ATEGTDKDV</th>
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</thead>
<tbody>
<tr>
<td>Hg2/pD7</td>
<td>........................................................................... KDV</td>
</tr>
<tr>
<td>hgam1</td>
<td>FFYQADDEHY IPRAVLLDE PRVIHSLNS PYAKLYNPEN IYLSEHGGGA</td>
</tr>
<tr>
<td>Hg2/pD7</td>
<td>FFYQADDEHY IPRAVLLDE PRVIHSLNS PYAKLYNPEN IYLSEHGGGA</td>
</tr>
<tr>
<td>hgam1</td>
<td>GNNWASGFSQ GEKIHEDIFD IIDREADGSD SLEGFLCHS IAGGTSGGLG</td>
</tr>
<tr>
<td>Hg2/pD7</td>
<td>GNNWASGFSQ GEKIHEDIFD IIDREADGSD SLEGFLCHS IAGGTSGGLG</td>
</tr>
<tr>
<td>hgam1</td>
<td>SYLLERLNDR YPKKLVQTYS VFPNDKEMSD VVVQYNSLL TLKRLTQAD</td>
</tr>
<tr>
<td>Hg2/pD7</td>
<td>SYLLERLNDR YPKKLVQTYS VFPNDKEMSD VVVQYNSLL TLKRLTQAD</td>
</tr>
<tr>
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<td>CLVVLNDNTAL NRIATDRLHI QNPSFSQINQ LVSTIMSAST TTLRYPGYMN</td>
</tr>
<tr>
<td>Hg2/pD7</td>
<td>CVVVLDNTAL NRIATDRLHI QNPSFSQINQ LVSTIMSAST TTLRYPGYMN</td>
</tr>
<tr>
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</tr>
<tr>
<td>Hg2/pD7</td>
<td>NDLIGLIASL ITPFRLHFLM TGYPTLTDQ SVASVRKTV LDVMRRLLQP</td>
</tr>
<tr>
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<td>KNVMVSTGRD RQTNHCYIAI LNIIGEVPDF TQVHSKQRI RERKLANFIP</td>
</tr>
<tr>
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</tr>
<tr>
<td>Hg2/pD7</td>
<td>WGPASIQVAL SRKSPYLPSA HRVSGLMMAN HTSIISSLFES SCQFDKLRR</td>
</tr>
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<td>REAFLEQFRK EDMFKNDFDE MDTSREIVQQ LIDEYBAATR PDYISWGTQE</td>
</tr>
<tr>
<td>Hg2/pD7</td>
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<tr>
<td>hgam1</td>
<td>Q2VPQDRGPS SALLVGPSPA ZLTTPSEHRS GTSRISFS... YTWTLCW</td>
</tr>
<tr>
<td>Hg2/pD7</td>
<td>Q2FPS...PL LLLLLDGHS LDHACSLZPS PTSWTTLGS SPARELVLLP</td>
</tr>
<tr>
<td>hgam1</td>
<td>PANTFTS.PL MRLFIFNKAL ............</td>
</tr>
<tr>
<td>Hg2/pD7</td>
<td>PFHALTLPML VQLZZSNKAW LSVKKKKK</td>
</tr>
</tbody>
</table>

Figure 8.
pDOW12. Although it is possible that the insert resulted from tissue-specific alternative splicing, it occurs in a highly conserved region of the γ-tubulin sequence.

Interestingly, this insert appears to render the Hy2 gene toxic and lethal to S. pombe. Horio and Oakley (1994) found that expression of a Hy1 cDNA supported the growth of S. pombe cells that lacked endogenous γ tubulin. To determine if this was the case for Hy2EST, I cloned Hy2EST into plasmid pAS248 such that its expression was driven by the S. pombe alcohol dehydrogenase (ADH) promoter. This promoter is constitutive and moderately strong. The resulting plasmid, pASHy2, was sent to Dr. Tetsuya Horio. Dr. Horio transformed pASHy2 into S. pombe strain NC377, selecting for the leucine marker on pASHy2. NC377 is a diploid strain which carries one functional γ-tubulin gene and one γ tubulin disrupted by a URA4+ gene. Only a few transformants were obtained compared to hundreds of transformants with control DNA. Dr. Horio suggested that the small number of transformants is significant and it seems likely that expression of pASHy2 is toxic to S. pombe.

But for the 27 bp insert in Hy2EST, the coding regions of Hy1 and Hy2 share 94.6% nucleotide identity and 97.3% amino acid identity (Figure 11). Many of the base pair differences that exist between the two genes are in the third position of the codon and cause no amino acid differences. Interestingly, eight of the twelve amino acid differences observed between the two sequences are in the C-terminal 61 amino acids of the proteins. The 3' UTRs of the two genes share 54% nucleotide identity (Figure 12). The poly(A)tail
of Hγ1 is 191 bp 3' to the stop codon and the poly(A) tail of Hγ2 is 204 bp 3' to the stop codon.

**Somatic Cell and Radiation Hybrid Mapping of Hγ2**

I designed two combinations of primers that specifically amplify 224 bp and 145 bp regions of Hγ2 (see Figure 18 in Chapter 3). These primers were given to Dr. Ralph Krahe's lab (Dept. of Human Cancer Genetics, OSU) who used them to amplify genomic DNA from three different chromosomal panels: (1) a human-CHO multi-chromosomal hybrid mapping panel, (2) a human-CHO mono-chromosomal panel, and (3) a GB4RH radiation hybrid panel. Analysis of the multi-chromosomal panel revealed that Hγ2, like Hγ1, maps to Chromosome 17. PCR analysis of the mono-chromosomal mapping panel unambiguously confirmed that Hγ2 maps to chromosome 17.

Further refinement of Hγ2's chromosomal location using the radiation hybrid panel indicated that Hγ2 maps to 17q12-21 a region that includes the Hγ1 gene (see Figure 13) (Friedman *et al.*, 1995; Rommens *et al.*, 1995; Couch *et al.*, 1995; Brody *et al.*, 1995). Because of these results we wanted to map the genes more precisely relative to each other. Since Dr. Joanna Rommen's lab had mapped a Hγ1 cDNA to a 700 kb YAC, HSD2 (Rommens *et al.*, 1995), I obtained a strain containing this YAC from Dr. Rommens and isolated DNA from the strain. Dr. Krahe's lab used the Hγ2 specific primers to determine if Hγ2 is also located on this 700 kb YAC. As is shown in Figure 14, the primer combinations amplified Hγ2 fragments. These results indicate that Hγ1 and Hγ2 are within
Figure 9: The nucleotide sequence of Hγ2EST and its predicted amino acid sequence.

The Hγ2EST cDNA is 1857 base pairs in length and contains the entire Hγ2 coding region as well as a 27 base pair in-frame insert within the coding region of the gene. The cDNA contains 244 base pairs of the 5' UTR and 232 base pairs of the 3'UTR. The clone ends at the poly(A)tail which is preceded by a polyadenylation signal (AATAAA) that is underlined in the figure. The start and stop codons are in bold face type.
(To be continued)

Figure 9.

82
(Figure 9 continued)

1150/301
gat gtc atg agg cgg ctc ctg cag ccc aag aac gtc atg gtc tcc aca ggc cga gac cgc
asp val met arg arg leu leu gln pro lys asn val met val ser thr gly arg asp arg
1210/321
cag acc aac cac tgc tac atc gcc atc atc aac atc cag gga gag gtc gac ccc acc
gln thr asn his cys tyr ile ala ile leu asn ile ile gln gly glu val asp pro thr
1270/341
cag gtc cac aag agc ctc gtc agg atc cgg gaa cgg aag ttt gcc aac ttc atc ccg tgg
gln val his lys ser leu gln arg ile arg glu arg lys leu ala asn phe ile pro thr
1330/361
ggc ccc gcc agc atc cag gtc gcc ctc tgg agg aag tct ccc tac cty ccc tgg gcc cac
gly pro ala ser ile gln val ala leu ser arg lys ser pro tyr leu pro ser ala his
1390/381
cgg gtc agc ggg ctc atg atg gcc aac cac acc agc atc tcc tgg ctc tgg ctt gaa agt tcc
arg val ser gly leu met met ala asn his thr ser ile ser ser leu phe glu ser ser
1450/401
tgc cag cag ttt gac aag ctc cgg aag cgg gat gcc ttc ctc gag ctc gtt gaa gtt ctc
cys gln gln phe asp lys leu arg lys arg asp ala phe leu glu gln phe arg lys glu
1510/421
gac atg ttc aag gac aac ttt gat gac atg gac agg tct agg gag gtt gag ctc gac
asp met phe lys asp asn phe asp glu met asp arg ser arg glu val val gln glu leu
1570/441
att gat gac ttc cat gcc gcc acc cag cca gcc tac att tcc tgg gcc acc cag gag cag
il e asp glu tyr his ala ala thr gln pro asp tyr ile ser thr pro thr glu gln glu
1630/461
tgatccctcccccaacttaccttctctctctctctctcagatggtaaccagcgcctctgacccttgtctctgaccctga
cp
ccacccatggcaaccccctctttgcttcctctcagcggcgtagctggtctctctctcagcggcgtacttt
taatgtctttgctctgtaataaagggataaagaccttggtctcagttataa

83
Abbreviations: **ADH**-alcohol dehydrogenase promoter; **LEU**-leucine selectable marker; **Ps**-PstI site; **Bm**-BamHI site; **Sm**-SmaI site; **Sc**-SacI site; **Bgl**-BglII ends of Hy2 specific primers.

The coding region plus 205 bp of the 3'UTR of Hy2EST was amplified from plasmid pHγ2EST with primers containing BglII ends. Following amplification the Hy2EST PCR product was digested with BglII. In parallel, plasmid pAS248 was digested with BamHI; the BamHI site is in the polylinker of pAS248. The BglII-digested Hy2EST PCR product was ligated into the BamHI-digested pAS248 downstream of the ADH promoter. The resulting plasmid was named pASHγ2.
Amplify the coding region plus 205 bp of the 3' UTR of \( H_2 \)EST located in \( pH_2 \)EST with \( H_2 \) specific primers containing BglIII ends.

Digest \( H_2 \)EST PCR product with BglIII.

Ligate \( H_2 \)EST into pAS248. The new plasmid construct is called pASHy2.

Figure 10.
Figure 11: A comparison of the predicted amino acid sequences of Hγ2EST and Hγ1.

Abbreviations: Hgam1-predicted amino acid sequence of Hγ1; Hg2EST-predicted amino acid sequence of Hγ2EST.

The coding regions of Hγ1 and Hγ2EST (minus the 27 bp insert that is underlined in this figure) share 97.3% amino acid identity. Only twelve amino acid differences exist between Hγ1 and Hγ2 and eight of the differences are clustered in the C-terminal 61 amino acids of the proteins. Most of the nucleotide differences that exist between the two genes occur in the third position of the codon thus enabling the amino acid sequences of the two genes to be highly conserved. Amino acid differences are marked by asterisks.
Figure 11.
Figure 12: A comparison of the nucleotide sequences in the 3' UTR regions of Hγ1 and Hγ2.

Abbreviations: Hgam1-Hγ1 3' UTR nucleotide sequence; Hgam2-Hγ2 3'UTR nucleotide sequence.

The 3' UTR's of Hγ1 and Hγ2 share 54% nucleotide identity. The polyadenylation signal (AATAAA) of both sequences is underlined. The poly(A)tail of Hγ2 is 204 base pairs 3' to the stop codon.
Figure 12.
Figure 13: Schematic diagram of human chromosome 17 showing Hγ1, BRCA1, and Hγ2.

This diagram of human chromosome 17 shows the position of Hγ1 in relation to the BRCA1, breast cancer susceptibility gene at region q21. Hγ2 maps within the 700 kb region that is magnified and shown to the left of the bracket.
Figure 13.
a 700 kb region of DNA that is located at region q21 on chromosome 17. Currently, Dr. Krahe's lab is using a high resolution (50-100 kb resolved) TNG radiation hybrid map to refine the mapping of Hγ1 and Hγ2.

**HγF is a fragment from a γ-tubulin pseudogene**

No HγF cDNAs were found in our probing of a HeLa cDNA library. This could mean that the HγF sequence is from a gene expressed at very low levels in HeLa cells, that the HγF sequence is from a pseudogene, or that HγF is a PCR amplification artifact (i.e. not of human origin). To determine if HγF is of human origin I designed primers, HGEN01B, HGEN02E, and HGENOR2 (underlined in Figure 15) that are specific for HγF (specificity was tested using plasmids containing Hγ1 and HγF) to use in PCR analyses of HeLa genomic DNA and human patient genomic DNA. PCR analysis was carried out as described in the Materials and Methods and results are shown in Figure 16. Fragments of the sizes expected for HγF (a 761 bp HGEN01B/2E band and a 576 bp HGEN01B/R2 band, Figure 16) were amplified from human patient genomic DNA and HeLa genomic DNA. These results verified that Dr. Shraudolf's original clone was really of human origin. I decided to sequence the 576 bp human genomic DNA PCR product to verify that the primers used were amplifying a subset of the HγF clone. Sequence analysis of both strands of the clone revealed that this clone is identical to the analogous region in the 930 bp-HγF clone.
DNA was isolated from YAC clone HSD2 and amplified with two \(\text{H}2\) specific primer combinations. The 224 bp \(\text{H}2\) fragment in lanes 2, 3, and 5 was amplified with HUGAM1-3F1 and HUGAM3R1, while HUGAM1-3F1 and HUGAM3R2 were used to amplify a 145 bp fragment of \(\text{H}2\) shown in lanes 8, 9, and 11. A \(\phi X/\text{HaeIII}\) molecular weight marker is shown in lane 1 and a 100 bp ladder is shown in lane 12. The positive amplification of \(\text{H}2\) from HSD2 YAC DNA, using both combinations of primers, is shown in lanes 5 and 11 respectively. Lanes 2, 3, 8, and 9 show positive amplification of human genomic DNA used as a positive control for amplification conditions.
Figure 14.
Figure 15: A comparison of the predicted amino acid sequences of HγF and Hγ1 with the HγF specific primers underlined.

Abbreviations: Hgam1-predicted amino acid sequence of Hγ1; HgamF-predicted amino acid sequence of HγF.

One common forward primer, HGENO1B, and two reverse primers, HGENO2E and HGENOR2 were designed to amplify fragments of HγF. The primers were tested for specificity of amplification and shown to specifically amplify HγF DNA and not Hγ1 DNA (data not shown). Both of these primer combinations were used to amplify HγF form HeLa genomic DNA and human genomic DNA. Dashes indicate that the amino acids in the HγF sequence are conserved. The asterisk represents a gap in the amino acid sequence of Hγ1 to compensate for an insertion in the HγF sequence.
**Figure 15.**

<table>
<thead>
<tr>
<th>Hgeno1B</th>
<th>Hgamf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hgam1</td>
<td>MPREIITLQLGQCGNQIGFEFKQLCAEHGISPEAIVEFATEGTDKDVFFYQADDEHY</td>
</tr>
<tr>
<td>Hgamf</td>
<td>G---------T---------KGM---------H----I---------</td>
</tr>
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<td>Hgam1</td>
<td>IPRAVLLDLEPRVHIHILNSPYAKLYNPEN1YLSHGGAGNNWSASGFSSQGERKHEFD</td>
</tr>
<tr>
<td>Hgamf</td>
<td>----------------------------------------R---------R---------R---------</td>
</tr>
<tr>
<td>Hgam1</td>
<td>IIDREADGDSLEGFVLCHSIGTTGSGLGSYGLERLNDRPKQLVQTYTSSFRPMQDEMSD</td>
</tr>
<tr>
<td>Hgamf</td>
<td>-T-Q------N-D------QF---------W---------------------K--N</td>
</tr>
<tr>
<td>Hgeno2R</td>
<td>Hgam1</td>
</tr>
<tr>
<td>Hgamf</td>
<td>----------------------------------------E---------H---------Q---------T---------I---------</td>
</tr>
<tr>
<td>Hgeno2R</td>
<td>Hgam1</td>
</tr>
<tr>
<td>Hgamf</td>
<td>I------S------D--------W--------Q--M--------W--------W--------</td>
</tr>
<tr>
<td>Hgam1</td>
<td>KNVMVSTGDRQTHCYAILNNIQQGEVDPTQVKHELQRLAQTRKLRKAPFPWFPGASIQVAL</td>
</tr>
<tr>
<td>Hgamf</td>
<td>------------------------S--------T--------</td>
</tr>
<tr>
<td>Hgam1</td>
<td>SRKSPYLPASRVSGLMNHNTSISSLFPERTCRQYDKLRKREAFLEQFRKEDMFKDNFDE</td>
</tr>
<tr>
<td>Hgam1</td>
<td>NDSREIVQQLIDEHYHAATRPDYSWGTQE</td>
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</table>
To shed light on HγF, we used the HγF sequence in a BLASTN search of the Washington University (St. Louis) Human Genomic Sequencing Center database. Sequences corresponding to HγF were found on two PAC clones (H_DJ1165K10.contig74 and H_DJ1022I14.contig59)(see Figure 17), both from chromosome 7. The two HγF-like sequences differ by only 2 bp (a GC in one sequence is CG in the other) in the region of interest. I surmised that these two sequences are of the same genomic region and the 2 bp differences are simply a sequencing error. Analysis of the two genomic clones reveals that they contain a γ-tubulin-like sequence that lacks introns. A functional γ-tubulin cDNA can not be expressed from these sequences for two reasons. First, there is no initiation codon in either genomic sequence. The codon corresponding to the initiation codon has been mutated from ATG to ATA. Second, the genomic fragment contains a one bp deletion and a 5 bp insert that disrupts the γ-tubulin reading frame. Consequently, I have concluded that the genomic sequence from which HγF originated is a γ-tubulin processed pseudogene.

Further inspection of the sequences suggests that although the sequences have diverged, this pseudogene was probably derived from Hγ1. The Hγ1 cDNA and the pseudogene share 92.4% nucleotide identity in the region corresponding to the coding region of the Hγ1 cDNA, 91.4% identity in the 187 bp region corresponding to the Hγ1 3'UTR and 92.0% identity in the 25 bp of the 5' UTR available for Hγ1 (Figure 17). In comparison, the Hγ2 cDNA and the pseudogene share 41.6% nucleotide identity in the 5'
Figure 16: PCR amplification of \textit{HyF} from HeLa cell and human genomic DNA using \textit{HyF} specific primers.

(16a) \textit{HyF} specific primers HGENO1B and HGENO2E were used to amplify a 761 bp fragment from HeLa genomic DNA (Lane 1) using the parameters given in the materials and methods. A 123 bp ladder (BRL) molecular weight marker is shown in lane 2. 

(16b) A 576 bp and a 761 bp fragment is shown in lanes 1 and 2 respectively. A plasmid named pIBI-\textit{γF}, that contains the 930 bp \textit{HyF} sequence, was used with both primer combinations as a positive control of amplification and the results are shown in lanes 3 and 4. Lane 5 contains the no DNA control for PCR contamination.
Figure 16.

a. 761bp
   123bp
   HγF

b. 761bp
   576bp
   100bp
   HγF
   HγF
   pIBI-γF
   pIBI-γF
   100bp
   No DNA
Figure 17: A comparison of the nucleotide sequence of human genomic clone "DJ1165k" with Hγ1, and HγF.

Abbreviations: Hgam1-Hγ1 sequence; Hgam2-Hγ2 sequence; HgamF-HγF sequence; DJ116K-DJ1165k genomic sequence.

The nucleotide sequence of human genomic clone DJ1165K is compared to the cDNA sequence of Hγ1, and the nucleotide sequence of HγF. The genomic clone lacks a proper initiation codon (underlined in the figure), and does not contain any introns within the 1351 base pairs corresponding to the Hγ1 coding region. The Hγ1 cDNA and DJ1165K share 92.4% nucleotide identity in the region corresponding to the coding region of Hγ1 and 91.4% nucleotide identity in the region corresponding to the Hγ1 3'UTR.
Figure 17.
(Figure 17 continued)

<table>
<thead>
<tr>
<th>hgaml</th>
<th>ACGAGATGAG CGATGTGGTG GTCCAGCCTT ACAATTCACT CCTCACACTC</th>
</tr>
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<tbody>
<tr>
<td>hgamf</td>
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</tr>
<tr>
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</tr>
<tr>
<td>hgamf</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>hgamf</td>
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</tr>
<tr>
<td>DJI165</td>
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<tr>
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<tr>
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(continued)
(Figure 17 continued)

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DJ1165  GCCCCTGTCG AGGAAGTCTC CTTACCCTGCC CTTGGCCCAC CGGGTCAGGG

hgaml  GGCTCATGAT GGCCAACCAC ACCAGACTTT CCTGGCTTGC GAGAGAGACC
hgaf  ...................................................................... ......................................................................
DJ1165  GGCTCATGAT GGCCAACCAC ACCAGACTTT CCTGGCTTGC GAGAGAGACC

hgaml  TGTGCGCCAGT ATGACAAGCT GGTAAGCTGG GAGCGCTTCC GAGAGAGCTT
hgaf  ......................................................................................................................................................
DJ1165  TGTGCGCCAGT ATGACAAGCT GGTAAGCTGG GAGCGCTTCC GAGAGAGCTT

hgaml  CCGCAAGGAG GACATGTTCA AGGACCCAGT TCTGACTGAC GACACAGAC
hgaf  ......................................................................................................................................................................
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hgaml  GGCCAGACTA CACCTCTTGG GGCACCACAG GAGCTCTCT CGAGCTCGA ACAGACTGAC
hgaf  .......................................................................................................................................................................
DJ1165  GGCCAGACTA CACCTCTTGG GGCACCACAG GAGCTCTCT CGAGCTCGA ACAGACTGAC

hgaml  AGGGGACCCT CACCTCTTGG GGCACCACAG GAGCTCTCT CGAGCTCGA ACAGACTGAC
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hgaml  CACCCCCTCA GAGCACAGAT CAGGGACCTC ACGTCTTCC CACCTCTTCC
hgaf  .......................................................................................................................................................................
DJ1165  CACCCCCTCA GAGCACAGAT CAGGGACCTC ACGTCTTCC CACCTCTTCC

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hgaf  .......................................................................................................................................................................
DJ1165  CATGACTCT CAGTTTCCTC GCAAAACATC TACTCTTCC TCTTACGTAG

hgaml  CTATTTAATCT TTAATAAGAC ACTGGG
hgaf  ..............................................................
DJ1165  CTATTTAATCT TTAATAAGAC ACTGGG

103
UTR, 89.6% identity in the coding region and 45.1% identity in the 3'UTR.

**Discussion**

Southern analyses indicate that there are probably no more than three \( \gamma \)-tubulin sequences in the human genome and three \( \gamma \)-tubulin sequences have now been identified: (1) \( H_\gamma 1 \), the first human \( \gamma \)-tubulin gene isolated by Zheng et al., (1991a,b); (2) \( H_\gamma 2 \), the second human \( \gamma \)-tubulin gene which I report in this study; and (3) a \( H_\gamma 1 \) processed pseudogene corresponding to sequences in \( H_\gamma F \) and two PAC clones. Consequently, I conclude that all the \( \gamma \)-tubulin sequences in *Homo sapiens* have now probably been identified.

The predicted products of \( H_\gamma 1 \) and \( H_\gamma 2 \) share 97.3% amino acid identity. The high amino acid identity shared between these two \( \gamma \)-tubulin cDNAs also exists between the pairs of \( \gamma \)-tubulin genes that have been isolated from *Arabidopsis thaliana* (Liu et al., 1994), *Zea mays* (Lopez et al., 1995), *Paramecium tetraurelia* (Ruiz et al., 1999), and *Euplotes octocarinatus* (Genbank, 1999), each of which share greater than 98% amino acid identity.

In order to obtain a full-length \( H_\gamma 2 \) clone, I have searched the EST database and obtained a \( H_\gamma 2 \)EST clone that contains the entire coding region of \( H_\gamma 2 \) as well as the 5'UTR and 3'UTR sequences to the poly(A)tail of the cDNA. Comparison of \( H_\gamma 2 \)EST to our four \( H_\gamma 2 \) cDNAs has revealed that \( H_\gamma 2 \)EST is identical to pDOW7 and pDOW21.
except for a 27 bp in-frame insert that is located in a highly conserved region of the γ-
tubulin sequence. Interestingly, this insert begins at the same position as a 5' intron splice
site that has been conserved in the genomic γ-tubulin clones identified in A. nidulans, S.
pombe and Ustilago violacea (Oakley and Oakley, 1989; Horio et al., 1991; Luo and
Perlman, 1993). Therefore, this insert may actually be part of an intron that was
misspliced. Unfortunately no γ-tubulin genomic clones have been sequenced from higher
eukaryotes, therefore there is no information on intron position in these organisms.

Rommens et al. (1995) have reported that Northern blot analysis shows that a Hy1
cDNA probe hybridizes to a doublet (1.8 kb and 1.85 kb) of γ-tubulin transcripts from total
RNA isolated from the frontal cortex of adult brain. The γ-tubulin probe hybridized to a
single 1.8 kb transcript when Caco-2 intestinal cell line, lymphoblast, HL60 and breast
carcinoma cell line total RNAs were probed. Rommens and colleagues (1995) suggest that
brain tissue may contain two γ-tubulin mRNAs produced by alternative splicing or
differential polyadenylation. Since the Hy2EST clone originated from a 73 day post-natal
infant brain cDNA library, the 27 bp insert may have resulted from brain-specific
alternative splicing. However, it is important to note that the 27 bp insert would cause a
nine amino acid insert in a highly conserved region of γ tubulin. In addition Dr. Tetsuya
Horio has found that the transformation of a S. pombe strain, lacking endogenous γ
tubulin, with plasmid pASHy2 is toxic. The 27 bp insert in pASHy2 may alter the Hy2
protein in a way that causes it to create a dominant negative effect when expressed in S.
pombe cells. Since Hγ2EST's function has only been tested in S. pombe the possibility exists that this form of γ tubulin is only found in specific human tissues such as brain tissue.

We (our lab in collaboration with Dr. Krahe's lab) have used PCR amplification with Hγ2-specific primers to map Hγ2 to the same chromosomal interval and, eventually to the same 700 kb YAC as Hγ1. It is interesting that the Hγ2 was not detected on the YAC carrying Hγ1 since this YAC has been analyzed by several labs involved in the search for the breast cancer susceptibility gene. The entire YAC was extensively analyzed and other novel and previously identified genes were found. Since these investigators used YAC and cosmid clones to screen cDNA libraries to find expressed genes that map to the YACs, the Hγ2 cDNA may have simply been overlooked or Hγ2 may not be expressed at detectable levels in the tissue or cell lines from which the cDNA was derived (i.e. human mammary gland, human breast, human breast cancer cell lines a human ovary λgt11 cDNA libraries, Rommens et al., 1995). Note that Hγ2 is expressed in ovaries (see Chapter 3). It is also possible, given the similarities of Hγ1 and Hγ2, that Hγ2 cDNAs were mistakenly identified as Hγ1 cDNAs.

Finally analysis of the HγF γ-tubulin clone has shown that it is a processed pseudogene of Hγ1. This is the first identification of a γ-tubulin pseudogene in any organism and likely completes the family of γ-tubulin sequences in humans.
CHAPTER 3

ANALYSIS OF THE EXPRESSION PATTERNS OF γ-TUBULIN GENES
IN HOMO SAPIENS

Introduction

After multiple γ-tubulin genes were identified in D. melanogaster, Oakley (1994) suggested that one of the future directions of γ-tubulin research would be to determine the number of γ-tubulin genes expressed in animals and plants, their patterns of expression in these organisms and whether sequence differences between γ tubulins within a species correlate with functional differences. While multiple γ-tubulin isoforms exist in six species other than humans, the expression patterns of only two of these species (Drosophila melanogaster and Arabidopsis thaliana) have been studied. Analysis of the expression patterns of the two D. melanogaster γ-tubulin proteins (γTUB23CD and γTUB37CD) (using antibodies specific for each protein) reveals that they are differentially expressed. γTUB23CD is zygotically expressed in male and female flies during all stages of development, while γTUB37CD is expressed in ovaries and the developing egg chambers during oogenesis and in developing embryos (Wilson et al.; 1997). Northern blot analysis
of the two \(\gamma\)-tubulin genes in *Arabidopsis* reveals that both genes are expressed in whole seedlings, roots, flowers, and suspension cell cultures (Liu et al., 1994). In both of these cases it is difficult to determine the relationship between the expression patterns of the two genes and their functions. Although multiple \(\gamma\)-tubulin isotypes exist, specific \(\gamma\)-tubulin isotype classes have not been identified among the species in which multiple \(\gamma\)-tubulin genes have been identified.

In Chapter 2, I reported the identification of a second \(\gamma\)-tubulin gene, \(H\gamma_2\), in *Homo sapiens*. In this chapter I have chosen to investigate the expression patterns of \(H\gamma_1\) and \(H\gamma_2\) in ten different human cell types using multiplex reverse transcription PCR (RT-PCR). Because \(H\gamma_1\) and \(H\gamma_2\) cDNA share 94.6\% base pair identity within their coding regions I was not able to find areas in their coding regions that were sufficiently different to allow synthesis of primers that could be used for specific amplification of the two sequences. To circumvent this problem I have designed specific reverse primers from sequences that lie within the 3' UTR of each gene to use in multiplex reverse transcription (RT) reactions. A forward primer, with a sequence common to \(H\gamma_1\) and \(H\gamma_2\), has been designed and used in combination with each reverse primer to give specific amplification of the two sequences. Multiplex RT-PCR has revealed that both \(H\gamma_1\) and \(H\gamma_2\) are expressed in all the tissues analyzed, although the two genes may not be expressed to the same extent within a given tissue or among different tissues.
Materials and Methods

Strains and Media

As positive controls for multiplex RT-PCR analysis, total RNA was isolated from S. pombe stains expressing all or portions of Hy1 and Hy2. A haploid strain, AH004, carrying a disrupted γ-tubulin gene and a plasmid, pTH5, which expresses Hy1 was constructed by Dr. Tetsuya Horio (Horio and Oakley, 1994). An S. pombe haploid strain HM123 (h⁰, leu1-32) was used as a wild-type strain and RNA from this strain was used as a negative control. Strain HMHG2 expresses a portion of Hy2 (see plasmid pASHγ2F construction and S. pombe transformation below).

Strain HM123 was streaked to single colony on YPD (1% yeast extract, 2% peptone, 2% dextrose and 1.7% agar for solid medium) plates. YPD medium was inoculated with single colonies of HM123 for transformation purposes and for total RNA preparations (see below). Strain AH004 was grown on YPD supplemented with 70 μg/ml of adenine for total RNA preparations. Strain HMHG2 was grown on EMM minimal medium (BIO101, Inc.) to select for plasmid pASHγ2F which contains the S. pombe LEU2 gene.

Plasmid pASHγ2F Construction

Ten nanograms of purified plasmid pDOW7 (a Hy2 partial clone in pBS sk-) DNA were used as a template to amplify a 1214 bp region of Hy2 (see Figure 19). Thirty cycles
of amplification using 2.5 units of Taq DNA polymerase (Gibco BRL) were carried out using the components and amounts recommended in the Gibco BRL protocol. Hy2-specific primers with BglII ends [Helagam3F: gaagatctcatgaagacatgttgc and HGEST3R1: gaagatctctgacaaccaagctttat (Operon Technologies)] were used to amplify a 1214 bp region of the Hy2 cDNA in pDOW7 using the following thermocycling parameters: 94°C for 2 min; followed by 30 cycles of 94°C for 1 min and 55°C for 4 min. The Taq DNA polymerase was removed from the PCR reaction using Strataclean Resin (Stratagene) and then the sample was concentrated in a Microcon-30 microconcentrator (Amicon, Inc.). This sample was digested with BglII as directed by the manufacturer (New England Biolabs) and then purified from a 0.7% SeaplaqueGTG low melting point agarose gel (FMC) using Wizard PCR preparations (Promega life sciences).

Plasmid pAS248 (Toda et al., 1991) was digested with BamHI as directed by the manufacturer (New England Biolabs) (see Figure 19). The BamHI enzyme was removed from the sample using Strataclean Resin (Stratagene) and the DNA was concentrated in Microcon-30 microconcentrator (Amicon, Inc.). The digested sample was treated with 0.01 units of Thermosensitive Alkaline Phosphatase (TsAP) (Gibco BRL) in a 10 µl mixture containing TsAP buffer diluted to 1x concentration and supplemented with MgCl₂ to a final concentration of 3 mM at 65°C for 20 min and then inactivated by following the manufacturer’s protocol.

The 1214 bp Hy2, BglII-digested fragment was ligated into the BamHI site in a
small polycloning site immediately downstream of the *S. pombe* alcohol dehydrogenase promoter (Russell and Hall, 1983). Ligation mixes were used to transform TOP10 *E. coli* competent cells (Invitrogen) using the manufacturer’s protocol. Wizard DNA minipreps were performed using the manufacturer's protocol (Promega Life Sciences). Test digests were performed on the miniprep DNAs to determine if the ligation was successful. The correct plasmid was identified and named pASHy2F (F=fragment). A standard large scale plasmid preparation (Sambrook *et al.*, 1989) was performed and pASHy2F was purified twice using equilibrium centrifugation in CsCl-EtBr gradients. Purified pASHy2F was used to transform *S. pombe*.

*S. pombe* Transformation

Haploid wild-type *S. pombe* strain HM123 (*h*, *leu*-32) was transformed with pASHy2F as described in the Lithium Acetate Procedure II protocol found in the Fission Yeast Handbook (www.bio.uva.nl/pombe) with the following modifications. A 20 ml culture of strain HM123 was grown at 32°C to an OD<sub>595</sub> = 0.5. After cells were harvested and washed they were resuspended in 0.1M lithium acetate at a density of 4 x 10⁸ cells per milliliter. One microgram of plasmid DNA (pASHy2F or pAS248) was added to the cells. Cells were incubated in 50% PEG at 32°C for 1 hour and then they were washed in 1 ml of sterile ddH₂O. Cells were resuspended in 200 µl of sterile ddH₂O and 100-200 µl aliquots were spread onto EMM plates. Cells were allowed to grow 4-5 days at 30°C. A positive transformant was given the designation HMHG2 (Figure 19). HM123 was also
transformed with pAS248 as a positive control for transformation efficiency.

Plasmid pASHγ2F DNA was isolated from strain HMHG2 using the Plasmid Recovery protocol (procedure A) found in the Fission Yeast Handbook (www.bio.uva.nl/pombe). This DNA was amplified using the Hy2-specific primers Helagam3F and HGESTR1 and the conditions described previously (see Plasmid pASHγ2F construction above), in order to verify that strain HMHG2 contains the correct Hy2-containing plasmid DNA.

Isolation of Total RNA from *S. pombe* Strains

Total RNA was extracted from *S. pombe* strains HM123, AH004, and HMHG2 using the "hot" phenol extraction method of Aves *et al.* (1985) with the following modifications. Cells were harvested by centrifugation and then they were frozen in 50 ml Falcon tubes in a dry-ice/ethanol bath. The frozen cell pellet was lysed using the liquid nitrogen cryo-impacting method of Smucker and Pfister (1975). All three RNA samples were treated with RNase-free DNase I (BMB) using a standard DNase I treatment protocol found in Sambrook *et al.* (1989) to ensure that the samples were not contaminated with DNA prior to reverse transcription.

Isolation of Total RNA from HeLa cells and Human tissues

We used standard methods to grow HeLa cells in culture (Freshney *et al.*, 1992) for RNA extraction. All human tissues were obtained through the Cooperative Human Tissue Network (CHTN) from the Tissue Procurement Division of the Dept. of Pathology of the
OSU Medical Center and tissues were homogenized following the TRIzol protocol using a Tekmar SDT100EN Tissumizer (Tekmar Co.). Total RNA was extracted from human tissues (kidney, liver, stomach, ovary, colon, heart, lung and testicles) and HeLa cells using TRIzol reagent (Gibco BRL) as described in the manufacturer's protocol. The RNA from each tissue was treated with RNase-free DNase I (BMB) using the standard DNase I treatment protocol found in Sambrook et al. (1989).

RT-PCR Analysis

Total RNA (8 µg of each type of human tissue RNA or 1 µg of HM123 RNA or 1 µg of AH004 and HMHG2 RNA mixed together) was reverse-transcribed at 42°C for 1 hour using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV-RT) (Gibco BRL) in a final volume of 20 µl. A multiplex reverse-transcription approach was used in which Ηγ1, Ηγ2, and human β-actin reverse primers (listed below) were combined in the same tube to facilitate simultaneous reverse transcription of these RNAs. Total RNA, 10X amplification buffer (Sambrook et al., 1989), reverse primers (1 µM final), and ddH₂O were mixed and heated to 75°C for 5 min. Mixtures were placed on ice 5 min and then 10 units of rRNasin (Promega), dNTPS (each 1 mM final), and M-MLV-RT (200U) were added to the sample. After a one hour incubation at 42°C, the M-MLV-RT was inactivated at 95°C for 5 minutes.

Multiplex PCR was performed in the GeneAmp 2400 PCR System (Perkin Elmer) with 2.5 µl of each RT reaction in a 50 µl final volume containing 3 mM MgCl₂. Each
primer was used at 0.4 μM except for HuGAM1-3F1 which was used at 0.8 μM. Cold dNTPS were used at 0.4 mM each and 5 μCi of α-32P dATP (3000Ci/mmol, ICN) was used for each 50 μl reaction along with 2.5 units of Platinum Taq DNA polymerase (Gibco BRL). PCR parameters were 30 cycles of 1 min at 94°C and 4 min at 60°C, after an initial denaturation of 2 min at 94°C. The following nucleotide primers were synthesized for use in RT and PCR analysis by Operon Technologies: HUGAM1-3F1-common forward primer-aggacaactttgtgagatggaca; HUGAM1R2-γ1 specific reverse primer-gttttgcaggcaacagagagtc; HUGAM3R2-γ2 specific reverse primer-atctagaaggagaaggagtagtg; HUACTF1 β-actin forward primer-ttctacaaatgagctgcgtggct; HUACTR1 β-actin reverse primer-gcttcttcctaatgtcgacgca. The RT-PCR products were run on 3.5% agarose gels [Agarose LE, (BMB)] that were dried and placed in a Molecular Dynamics phosphor cassette for 18-20 hours. Images were analyzed with ImageQuant version 4.1 on a Molecular Dynamics 445SI Phosphorimager.

**Results**

**Rationale for Primer Design and Controls for RT-PCR**

The existence of two functional γ tubulin genes has raised the important question of whether these genes are differentially expressed in various human tissues. RNase protection assays and Northern blot analysis are often used to study the expression patterns of genes. Both approaches were problematic for examination of the expression of Hγ1 and
Hy2, however, because of the great sequence similarities of the mRNAs encoded by the two genes and because each of the two genes is likely to be expressed at relatively low levels. I chose, instead, to use two-step multiplex reverse transcription PCR (RT-PCR) (Chelly and Khan, 1994). In this technique multiple mRNAs from a tissue are first reverse transcribed and then an aliquot of the reverse transcription mixture is used for PCR with gene-specific primers.

In order to use RT-PCR analysis to analyze Hy1 and Hy2 expression patterns, it was necessary to design primers specific for each gene. The similarity of Hy1 and Hy2 sequences within the coding region precluded designing specific primers. However, the 3' UTR of the two genes were sufficiently divergent that specific primers were feasible. I chose to use a forward primer common to the coding region of Hy1 and Hy2 and two reverse primers, each specific for the 3' UTR of Hy1 or Hy2 (see Materials and Methods). This combination of primers should amplify a 252 bp fragment specific to Hy1 and a 145 bp fragment specific to Hy2 (see Figure 18). The specificity of the primers was first verified by PCR amplification of plasmids containing Hy1 and Hy2 cDNAs (results not shown). Neither primer combination falsely amplified Hy1 or Hy2 plasmid DNA. As a further and more important control, RT-PCR was carried out with the multiplex primer mixture on RNA isolated from three Schizosaccharomyces pombe strains. One strain (AH004) produced an Hy1 transcript, a second strain (HMHG2) (Figure 19) produced a
Figure 18: A comparison of the nucleotide sequences found in the 3' UTRs of Hγ1 and Hγ2.

Abbreviations: **Hgam1** - Hγ1 3'coding and 3'UTR nucleotide sequence; **Hgam2**-Hγ2 clone pDCW7 3' coding and 3'UTR nucleotide sequence.

A common forward primer, HUGAM1-3F1 (labeled HG1-3F1) and two specific reverse primers, HUGAM3R2 (labeled HG3R2) and HUGAM1R2 (labeled HG1R2) are underlined. Both reverse primers were used in multiplex reverse transcription reactions. Aliquots of the cDNAs were then used in PCR reactions in which HUGAM1-3F1 and HUGAM1R2 amplify a 252 bp Hγ1 fragment, while HUGAM1-3F1 and HUGAM3R2 amplify a 145 bp Hγ2 fragment.
Figure 18.
Figure 19: The construction of plasmid pASHγF and *S. pombe* strain HMGH2.

A 1214 bp region of the Hγ2 clone in pDOW7 was amplified using Hγ2 specific primers that contain BglII ends. The Hγ2 PCR product was then digested with BglII. In parallel, plasmid pAS248 was digested with BamHI. The BglII digested Hγ2 PCR product was ligated into the BamHI site of pAS248 downstream of a constitutive alcohol dehydrogenase promoter. The new plasmid was named pASHγ2F. Wild-type *S. pombe* strain HM123 was transformed with pASHγ2F to create strain HMGH2. Total RNA was isolated from this strain and used as a positive control for RT-PCR analysis of Hγ2's expression in various human tissues.
Amplify a 1214 bp region of Hγ2 using primers with BglII ends.

Digest Hγ2-BglII PCR product with BglII.

Digest pAS248 with BamHI.

Ligate Hγ2EST into pAS248. The new plasmid construct is called pASHγ2.

Transform strain HM123

S.pombe strain HMHG2

Figure 19.
transcript that covers most of the Hy2 sequence (including the 3' end and 3' UTR), and a third strain (HM123) only transcribed the endogenous S. pombe γ-tubulin gene. As anticipated, only the 252 bp fragment specific to Hy1 was amplified from AH004, only the 145 bp fragment specific to Hy2 was amplified from HMG2 and neither fragment was amplified from HM123 (Figure 20). When RT-PCR was carried out on a mixture of AH004 and HMG2 RNA, both fragments were amplified (Figure 20). These results indicated that multiplex RT-PCR with these primers under the conditions used is sufficiently sensitive and specific to determine if Hy1 and Hy2 are expressed in various tissues.

Expression Patterns of γ-tubulin Genes in Human Tissues

I isolated total RNA from the ten tissues shown in Figure 21 and carried out RT-PCR with the Hy1 and Hy2 primer mixture. The mixture also included primers specific for human β-actin to serve as an internal control for the quality of RNA in the samples and as an internal control for the RT-PCR reactions. Examination of the expression patterns of Hy1 and Hy2 (Figure 21) revealed that both genes are expressed in all the tissues examined, but there is some apparent variation in the levels of expression of the two genes. Although RT-PCR is, at best, semiquantitative, the relative amounts of Hy1 and Hy2 seem to be different in some tissues and the level of expression of each of them seems to vary among tissues. For example, Hy1 and Hy2 are both expressed in testicular tissue (Figure 21), but if the testicular Hy1 band is compared to the Hy1 band from HeLa cells (Figure
Figure 20: PCR analysis of total RNA isolated from three *S. pombe* strains used as controls for RT-PCR.

The AH004/HMHG2 labelled lane contains the multiplex RT-PCR products of *S. pombe* strains AH004 and HMHG2 that express Hy1 and Hy2 respectively. These RT-PCR products served as positive controls for the PCR parameters used in the analysis of γ-tubulin expression patterns in various human tissues. The HM123 labelled lane is blank and served as a negative control because neither Hy1 nor Hy2 specific primers amplify the endogenous *S. pombe* γ-tubulin gene expressed by this strain. The Hy1 primer combination amplifies a 252 bp band and the Hy2 specific primer combination amplifies a 145 bp band. A 100 bp ladder (Gibco BRL) is also shown.
21, lane 1), the Hγ1 band seems more intense in HeLa cells. The HeLa cell γ-tubulin bands are more intense than bands from other tissues and may simply reflect the fact that HeLa cells are aneuploid. Conversely increased γ-tubulin expression in HeLa cells may be due to the presence of supernumerary centrosomes that are often found in tumor cells. The no-RT control reactions for each tissue (lanes 14-23), the HM123 negative γ-tubulin control (lane 24) and the no-DNA PCR control were all negative. Therefore the amplification products we obtained were not caused by contamination or amplification of tissue DNA.

**Discussion**

I have used multiplex RT-PCR to analyze the expression patterns of Hγ1 and Hγ2 in several human tissues. RT-PCR analysis has shown that both Hγ1 and Hγ2 are expressed at varied levels in all of the tissues assayed. A survey of γ-tubulin clones in the human EST database (www.ncbi.nlm.nih.gov/dbEST) has revealed that Hγ1 clones have been isolated from 27 different tissue/cell cDNA libraries including nine tumor cell libraries and a multiple sclerosis cell line, while Hγ2 clones have been isolated from 11 different tissue/cell cDNA libraries including two tumor cell libraries. This corroborates our findings that both Hγ1 and Hγ2 are widely expressed in specific human cell types and tissues.

Although Hγ1 and Hγ2 are highly homologous, they encode γ-tubulins with
Radioactive multiplex RT-PCR was conducted as described in the Materials and Methods. RT-PCR products were run on 3.5% agarose gels. Molecular Dynamics phosphor screens were exposed to gels 18-20 hours. Lanes 1-10 contain RT-PCR products from the Human tissues indicated above the lanes. Lane 11 contains the Hy1 and Hy2 positive control transcripts that were expressed in S. pombe strains AH004 and HMG3. Lanes 14-23 contain negative control reactions for each of the tissues found in lanes 1-10. These reactions lacked reverse transcriptase and were run to make sure samples were free from DNA contamination. S. pombe wild-type strain HM123 expresses endogenous γ tubulin. Lane 24 which contains the HM123 negative control, confirms the specificity of the Hy1 and Hy2 primers. A final PCR reaction (labelled No cDNA) was also run that lacked any cDNA in order to ensure that PCR solutions were not contaminated. The β-actin, Hy1, and Hy2 bands’ expected sizes are indicated.
slightly different sequences. Vertebrate β tubulins and mammalian α tubulins are classified into at least six "isotype classes", based on the conservation of their C-terminal 15 amino acids and conservation of their function, as is seen within the same and across different species (Sullivan and Cleveland, 1986; Lewis et al., 1985; Villisante et al., 1986). To date, no isotype defining motif has been identified in any of the species from which multiple γ-tubulin genes have been isolated. Thus while γ tubulins encode different isotypes, there is no evidence as yet for "isotype classes".

As has been previously mentioned, Hγ1 and Hγ2 share 97.3% amino acid identity. Why would an organism need two γ-tubulin isotypes, that encode nearly identical γ-tubulin proteins? These two very homologous genes may exist to encode proteins that perform specific functions in cells. Recent studies by Ruiz et al. (1999) have revealed that γ tubulin is required for basal body duplication and therefore may also be involved in centriole duplication. If only one γ-tubulin isotype is involved in the basal body duplication then there probably would be increased expression of this isotype in cells that are heavily ciliated such as lung endothelium and intestines or cells that contain basal bodies like sperm.

At the present time, however, there is no evidence for this functional specificity and it seems unlikely that the functional specificity of γ-tubulin is determined by such a small number of amino acid differences. In the case of Hγ1 and Hγ2 this functional specificity would have to be determined by the twelve amino acid differences that exist between the two genes.
The two genes could exist to facilitate regulation of the levels of γ-tubulin synthesis in different tissues. Our RT-PCR experiments suggest that this may be the case but RT-PCR is semi-quantitative at best and we must be cautious when interpreting the variations in levels of Hγ1 and Hγ2 message we see.

Finally, it is possible that the two γ-tubulin genes exist for simple functional redundancy. γ Tubulin is essential for viability in all organisms that have been studied and having two genes instead of one would greatly reduce the chance that a γ-tubulin mutation would lead to loss of viability.
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145


