

# Missense Mutations at Lysine 350 in $\beta$ 2-Tubulin Confer Altered Sensitivity to Microtubule Inhibitors in *Chlamydomonas*

Vincent D. Lee<sup>1</sup> and Bessie Huang

Department of Molecular Biology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, California 92037

**Two  $\beta$ -tubulin mutants of *Chlamydomonas reinhardtii*, *col<sup>R</sup>4* and *col<sup>R</sup>15*, were previously isolated in our laboratory. Each mutant expressed an acidic  $\beta$ -tubulin variant as a result of an alteration in the coding sequence of one of the two  $\beta$ -tubulin genes in *C. reinhardtii*, which in the wild type encode identical proteins. In this report, we describe the identity of the specific  $\beta$ -tubulin altered in the *col<sup>R</sup>* mutants and the precise nature of the genetic lesions. Hybrid selection of mutant poly(A)<sup>+</sup> RNA with cDNA probes specific for the two  $\beta$ -tubulins in *C. reinhardtii* indicated that both mutations resided in the  $\beta$ 2-tubulin gene. cDNA libraries were constructed with mutant poly(A)<sup>+</sup> RNA, and  $\beta$ 2-tubulin cDNA clones were isolated. Results of in vitro transcription of cloned  $\beta$ 2-tubulin cDNAs confirmed the identity of the altered genes. Sequencing of the entire coding regions of the  $\beta$ 2-tubulin cDNA clones revealed that the mutants carried different single-base substitutions in the same codon for the amino acid at position 350 in the  $\beta$ 2-tubulin sequence, effecting a change from a lysine to a glutamic acid in the *col<sup>R</sup>4* variant and to a methionine in the *col<sup>R</sup>15* variant. These changes in amino acids are consistent with the difference in the charge of the two variant polypeptides observed in isoelectric focusing. Because both the *col<sup>R</sup>4* and *col<sup>R</sup>15* mutations confer an altered sensitivity to a number of different microtubule inhibitors and herbicides, lysine 350 appears to be of functional importance in the structure of the tubulin molecule.**

## INTRODUCTION

In plants as well as in other eukaryotes, microtubules are composed of heterodimeric subunits consisting of  $\alpha$ -tubulin and  $\beta$ -tubulin, which are related but distinct polypeptides. In line with many eukaryotic proteins of central importance in cellular processes, tubulins are generally encoded in small, multigene families (for a review, see Cleveland and Sullivan, 1985). Although there is a remarkable conservation of  $\alpha$ -tubulin and  $\beta$ -tubulin in evolution, as confirmed at the primary sequence level, sequence microheterogeneity in clusters of variable and conserved regions has been documented for  $\beta$ -tubulin and, to a lesser extent, for  $\alpha$ -tubulin (for a review, see Sullivan, 1988). In addition to constituting the major structural component of the mitotic and meiotic spindles and cilia and flagella, microtubules participate in intracellular transport, and as a part of the cytoskeleton, they play a central role in the establishment and the maintenance of cell shape and of the spatial organization of the cytoplasm (for a review, see Dustin, 1984). In plants, microtubules serve in additional, specialized roles in cell division and cell expansion during development (for a review, see Gunning and Hardham,

1982). This functional multiplicity is paralleled by a diversity in properties: microtubules, depending on their source, differ in their modes of assembly, degrees of stability, patterns of post-translational modification, and constitutions of specific microtubule-associated proteins. Reconciling the relationship between microtubule function and heterogeneity and elucidating the spatial and temporal control of microtubule function and specificity are paramount in the understanding of microtubule biology.

One approach to the understanding of microtubule structure and function has been the isolation of mutations in the structural genes for tubulins (for reviews, see Cabral et al., 1984; Raff, 1984; Oakley, 1985). Analysis of such mutations has not only provided insights into the relationship between differential expression of tubulin genes and specific microtubule-dependent cellular processes (e.g., May et al., 1985; Matthews and Kaufman, 1987; Fuller et al., 1988), it has also provided a framework in which the functional properties of structural domains on the tubulin molecules can be assessed (e.g., Fridovich-Keil et al., 1987; Schatz et al., 1987; Huffaker et al., 1988; Katz and Solomon, 1988). In previous studies, we isolated two  $\beta$ -tubulin mutants of the unicellular green alga *Chlamydo-*

<sup>1</sup> To whom correspondence should be addressed.

*monas reinhardtii*, *col<sup>R</sup>4* and *col<sup>R</sup>15*, on the basis of an increased resistance to the growth-inhibitory effects of the microtubule depolymerizing drug colchicine (Bolduc et al., 1988). Although tubulin mutants have been isolated in a number of different organisms based on an altered sensitivity to microtubule inhibitors, *col<sup>R</sup>4* and *col<sup>R</sup>15* represent the first such mutants reported in an algal or higher plant system. This is significant because it has been demonstrated that plant tubulins differ from animal tubulins in some of their biochemical properties and in their sensitivity to different anti-microtubule drugs and herbicides (for a review, see Morejohn and Fosket, 1986).

The *col<sup>R</sup>4* and *col<sup>R</sup>15* mutants were initially identified by an altered  $\beta$ -tubulin content from electrophoretic analysis of tubulins isolated from their flagellar axonemes (Bolduc et al., 1988). As has been previously reported (Silflow and Rosenbaum, 1981; Youngblom et al., 1984), wild-type *C. reinhardtii* contains two  $\beta$ -tubulin genes,  $\beta$ 1 and  $\beta$ 2, which encode two identical polypeptides that are resolved as a single  $\beta$ -tubulin species in two-dimensional polyacrylamide gels (Lefebvre et al., 1980; Adams et al., 1981). In contrast to the wild type, both *col<sup>R</sup>* mutants were found to express an acidic  $\beta$ -tubulin variant assembled into the flagellar microtubules in a molar ratio approximately equal to that of a wild-type  $\beta$ -tubulin; the *col<sup>R</sup>4* variant  $\beta$ -tubulin migrated to a more acidic pI value than the *col<sup>R</sup>15* variant in isoelectric focusing (IEF). Analysis of the in vitro translation products of total poly(A)<sup>+</sup> RNA from the mutants indicated that the variant  $\beta$ -tubulins in the *col<sup>R</sup>* mutants were the results of mutations in the coding sequence of one of the two  $\beta$ -tubulin genes (Bolduc et al., 1988).

More recently, the two  $\beta$ -tubulin mutants have been found to express, in addition to resistance to colchicine, altered sensitivities to several chemically distinct microtubule inhibitors, including the antimetabolic herbicides pronamide and amiprofos-methyl and the dinitroanilines oryzalin, trifluralin, and profluralin (Schibler and Huang, 1987; Bolduc et al., 1988). In this report, we present data that identify the specific  $\beta$ -tubulin gene altered in the *col<sup>R</sup>4* and *col<sup>R</sup>15* mutants and the precise nature of the genetic lesions that give rise to the altered drug sensitivity phenotypes.

## RESULTS

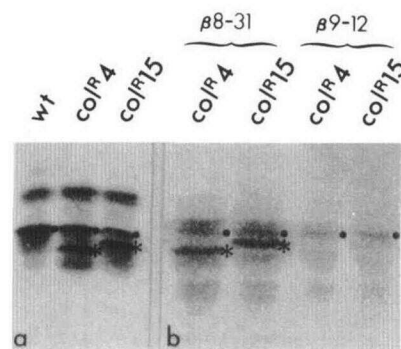
### Identification of Mutated Genes by Hybridization Selection

To identify the  $\beta$ -tubulin genes altered in the *col<sup>R</sup>* mutants, positive hybridization selection of mutant poly(A)<sup>+</sup> RNA was performed with full-length cDNAs for the two  $\beta$ -tubulin genes in *C. reinhardtii*. Although the two  $\beta$ -tubulin genes in this organism code for identical polypeptides with a very high identity in their nucleotide sequences for the coding

regions, they are quite dissimilar in their 5'-untranslated and 3'-untranslated regions. Two plasmids containing full-length cDNAs, pcf9-12 and pcf8-31, which have previously been reported (Youngblom et al., 1984) to show preferential hybridization to the transcripts for the  $\beta$ 1-tubulin and  $\beta$ 2-tubulin genes, respectively, were used. Figure 1b shows the resolution in a one-dimensional IEF slab gel of <sup>35</sup>S-methionine-labeled in vitro translation products of the selected RNAs. Under the appropriate washing conditions, we found that pcf9-12, which shows preferential hybridization to the  $\beta$ 1-tubulin transcript, appeared to select RNA that coded for the wild-type  $\beta$ -tubulin isoform, whereas pcf8-31, which shows preferential hybridization to the  $\beta$ 2-tubulin transcript, preferentially selected RNA that encoded the variant  $\beta$ -tubulin isoform. In Figure 1a, <sup>35</sup>S-labeled wild-type and mutant axonemal proteins, also resolved in a one-dimensional IEF slab gel, are shown for comparison. Taken together, then, the results shown in Figure 1 indicated strongly that in both *col<sup>R</sup>4* and *col<sup>R</sup>15* the wild-type  $\beta$ -tubulin isoform is the product of the  $\beta$ 1-tubulin gene and that the variant isoform is the product of the  $\beta$ 2-tubulin gene.

### Confirmation of Gene Identity by in Vitro Transcription and Translation

To confirm the identity of the  $\beta$ -tubulin genes altered in the *col<sup>R</sup>* mutants, we constructed cDNA libraries in  $\lambda$ gt11



**Figure 1.** Autoradiograms of IEF Slab Gels.

(a) Resolution of <sup>35</sup>S-labeled axonemal polypeptides isolated from wild-type 137c (wt) and strains carrying the *col<sup>R</sup>* mutations (*col<sup>R</sup>4* and *col<sup>R</sup>15*).

(b) Resolution of <sup>35</sup>S-methionine-labeled  $\beta$ -tubulin polypeptides synthesized in vitro from hybrid-selected poly(A)<sup>+</sup> RNA isolated from the *col<sup>R</sup>* mutants. Plasmid DNA containing either  $\beta$ 1-tubulin cDNA ( $\beta$ 9-12) or  $\beta$ 2-tubulin cDNA ( $\beta$ 8-31) was used for hybrid selection. The results of hybridization selection with  $\beta$ 8-31 were obtained by using wash conditions (1) and those with  $\beta$ 9-12 by wash conditions (2), as described in Methods (Hybridization Selection). The positions of the wild-type  $\beta$ -tubulin isoform are indicated with periods (.), and the positions of the mutant  $\beta$ 2-tubulin isoform are indicated with asterisks (\*).

phage vector with mutant poly(A)<sup>+</sup> RNA and isolated the  $\beta$ 2-tubulin cDNA clones. The cloned mutant  $\beta$ 2-tubulin cDNAs and the wild-type  $\beta$ 2-tubulin cDNA were each inserted into the transcription vectors pT7/T3-18 and pT7/T3-19, which carry the promoters for T7 and T3 phage RNA polymerases. The inserts in the vectors were oriented so that in vitro transcription with T7 RNA polymerase produced coding strands of RNA. Transcripts synthesized in vitro off the vectors were translated in a reticulocyte lysate system. Shown in Figure 2 are <sup>35</sup>S-methionine-labeled in vitro translation products of the transcripts synthesized in vitro. Wild-type  $\beta$ 2-tubulin cDNA produced a transcript that was translated in vitro into a polypeptide that comigrated with the wild-type  $\beta$ 2-tubulin found assembled into axonemal microtubules, whereas  $\beta$ 2-tubulin cDNAs from the *col*<sup>R</sup> mutants gave rise to the more acidic  $\beta$ -tubulin variants, the *col*<sup>R</sup>4 mutant  $\beta$ 2-tubulin cDNA product carrying a more acidic pI than that of the *col*<sup>R</sup>15 mutant. The migration pattern of these polypeptides corresponded to the observed mobility of the  $\beta$ -tubulins found assembled into wild-type and mutant axonemal microtubules.

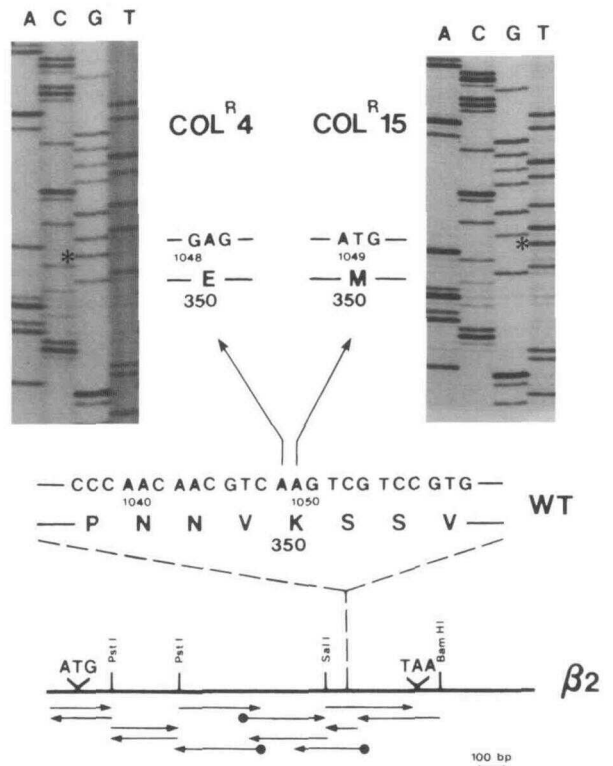
#### Localization of Lesions in Mutant $\beta$ 2-Tubulin Genes by cDNA Sequencing

To identify the molecular nature of the mutations, we sequenced the entire coding regions of the cDNA clones



**Figure 2.** In vitro Translation of RNA Synthesized from  $\beta$ 2-tubulin cDNA from Wild-Type 137c and Strains Carrying the *col*<sup>R</sup> Mutations.

RNA was synthesized from the pT7/T3 transcription vector containing  $\beta$ 2-tubulin cDNA and translated in a reticulocyte lysate in the presence of <sup>35</sup>S-methionine as described in Methods. Products of in vitro translation were resolved in a one-dimensional IEF gel. As a control, the transcription vector without insert DNA was processed identically in the experiment, and the result is as shown.



**Figure 3.** Sequencing of  $\beta$ 2-Tubulin cDNA from Strains Carrying the *col*<sup>R</sup> Mutations.

Shown is a schematic representation of *Chlamydomonas*  $\beta$ 2-tubulin cDNA with the positions of the initiation and termination codons indicated. Arrows at the bottom show the strategy of sequencing used. Plain arrows ( $\leftarrow$ ) indicate sequences obtained using an M13 17-mer universal primer. Arrows with filled circles ( $\leftarrow$ ●) denote sequences obtained using as primers synthetic oligonucleotides prepared against previously sequenced regions. A portion of the wild-type sequence around lysine 350 is expanded to show the nucleotides involved in the mutations. Numbers below the nucleotides indicate their positions within the coding sequence. Also shown are portions of the sequencing gels that pertain to the base changes detected in the mutant cDNA. Asterisks (\*) indicate the altered bases.

in both directions with the strategy shown in Figure 3. The results of sequencing the  $\beta$ 2-tubulin cDNAs from the mutants, when compared with the published coding sequence for  $\beta$ 2-tubulin in *C. reinhardtii* (Youngblom et al., 1984), revealed that the *col*<sup>R</sup> mutants carried single-base substitutions in the same codon in the  $\beta$ 2-tubulin sequence (Figure 3). In the case of *col*<sup>R</sup>4, there was a substitution of a guanine base for the adenine base at nucleotide 1048 of the coding sequence, which resulted in the change of the codon at this position from AAG to GAG, and thus in the substitution of an acidic glutamic acid for the basic lysine at amino acid 350. In the case of *col*<sup>R</sup>15, a thymidine

substituted for an adenine base at nucleotide 1049 of the coding sequence, effecting a change of the codon at that position from AAG to ATG and the consequent substitution of an apolar methionine for the basic lysine at the same amino acid position of 350. The amino acid changes were consistent with the difference in the charge of the two variant polypeptides observed in IEF, with the *col<sup>R</sup>4* variant polypeptide carrying a more acidic pI value than that of *col<sup>R</sup>15*.

### Conservation of Lysine 350 in $\beta$ -Tubulins of Higher Eukaryotes

The primary amino acid sequence of the  $\beta$ -tubulin in *C. reinhardtii* (Youngblom et al., 1984) exhibits a high degree of homology with  $\beta$ -tubulins from both animal and higher plant systems but a lower degree of sequence identity to yeast  $\beta$ -tubulin. Biochemical studies have provided evidence that both  $\alpha$ -tubulin and  $\beta$ -tubulin are organized into two functional domains, which differ in predicted secondary structure: an amino-terminal domain has been implicated in GTP binding, and a carboxyl-terminal domain has been shown to interact with microtubule-associated proteins, to bind  $\text{Ca}^{2+}$ , and to play a major role in the regulation of tubulin assembly (for a review, see Sullivan, 1988). Figure 4 shows an expanded region of the sequence surrounding lysine 350 in the  $\beta$ -tubulin of *C. reinhardtii*

		345		350		355	
<i>Chlamydomonas</i> $\beta 1, \beta 2$		V E W I P N N		V K S S V		C D I P P	
<i>Arabidopsis</i> <u>TUB1, TUB5</u>							A
<i>Arabidopsis</i> <u>TUB4</u>							
Corn $\beta 10$							T
Soybean $\beta 1$							
$\beta 2$			H				T
Chicken $\beta 1, \beta 2, \beta 3$							T A
$\beta 4, \beta 5$							V A
Human M40, $\beta 2, \beta 5$							T A
$\beta 4$							V A
Mouse M $\beta 2$ , M $\beta 4'$ , M $\beta 5$							T A
M $\beta 4$							A A
Pig $\beta$ (A, B)							T A
Sea Urchin $\beta 2, \beta 3$							T A
<i>Drosophila</i> $\beta 2$				C			T A
$\beta 3$							T A
<i>Trypanosoma</i>					I		
<i>Aspergillus</i> <u>BENA</u>		I					Q A L S
<u>TUBC</u>							T A L S M
<i>Neurospora</i> <u>TUB2</u>							Q T A L S
<i>Saccharomyces</i> <u>TUB2</u>							Q T A S V A
<i>Schizosaccharomyces</i> <u>NDA3</u>			D				L K A S V

**Figure 4.** Comparison of Amino Acid Sequences Surrounding Lysine 350 in  $\beta$ -Tubulins.

The region of the sequence surrounding lysine 350, which is replaced in the mutant  $\beta 2$ -tubulins in *Chlamydomonas* (Youngblom et al., 1984), is shown at the top.  $\beta$ -Tubulin sequences from a variety of species in the region around the lysine residue are included below for comparison (Cleveland and Sullivan, 1985; Sullivan and Cleveland, 1986; Silflow et al., 1987; Sullivan, 1988; GenBank Genetic Sequence Data Bank); only those amino acid residues that differ from the *Chlamydomonas* sequence are displayed.

with the analogous regions of the  $\beta$ -tubulin sequences from other species included for comparison. Lysine 350, which lies in the carboxyl-terminal region of the molecule that is enriched in predicted alpha-helical structures, is a conserved residue in the  $\beta$ -tubulins of higher eukaryotes, including plant and animal cells. In contrast, all fungal  $\beta$ -tubulins sequenced to date have a different amino acid residue at this position. Although the significance of this observation cannot be evaluated at this time, it should be noted that fungal tubulins have been found to differ from both plant and animal tubulins in their in vivo sensitivity and in vitro affinity to colchicine and other microtubule inhibitors (e.g., Morejohn and Fosket, 1986).

### DISCUSSION

In this paper, we have demonstrated that the variant  $\beta$ -tubulins expressed in the *C. reinhardtii* *col<sup>R</sup>4* and *col<sup>R</sup>15* mutants that give rise to an increased resistance to the growth-inhibitory effects of colchicine are products of different single-nucleotide changes in the codon for amino acid residue 350 in the  $\beta 2$ -tubulin gene. Although colchicine-resistant tubulin mutants have been previously identified in Chinese hamster ovary cells in culture (for a review, see Cabral et al., 1984), *col<sup>R</sup>4* and *col<sup>R</sup>15* represent the first such mutations in which the precise nature of the mutations has been defined.

The observation that both mutations represent different nonconservative substitutions for the same amino acid residue is striking. At the present time, it is unknown whether the codon for amino acid residue 350 is a preferred site for spontaneous or ultraviolet-induced missense mutations that confer resistance to colchicine, or whether lysine 350 represents a critical residue in the structure of the  $\beta$ -tubulin molecule for colchicine binding activity or microtubule assembly. In this regard, we have noted that in several lower eukaryotes, altered sensitivity to a different microtubule depolymerizing drug, benomyl, has been associated with conservative and nonconservative replacement of several different single amino acid residues in the  $\beta$ -tubulins. These include mutations in residue 167 in *Neurospora crassa* (Orbach et al., 1986), in residues 241, 318, and 391 in *Saccharomyces cerevisiae* (Thomas et al., 1985), and in residues 6, 50, 134, and 165 in *Aspergillus nidulans* (Jung et al., 1987).

In the case of the *col<sup>R</sup>* mutations in the  $\beta$ -tubulin gene, it is clear that the mutations do not result in the failure of the tubulins to form intradimer and interdimer bonds with other tubulin molecules to assemble functional microtubules. The variant  $\beta 2$ -tubulins are assembled into flagellar microtubules in a molar ratio approximately equal to that of a wild-type  $\beta 1$ -tubulin, and in the absence of colchicine the mutants show normal growth characteristics and microtubule-associated cell functions (Bolduc et al., 1988).

The *col<sup>R</sup>* mutations in the  $\beta$ 2-tubulin gene may confer an increased resistance to the effects of colchicine by specifically affecting the colchicine-binding affinity of the tubulin molecule. In this regard, we should note that lysine 350 lies in proximity to 1 of the 2 cysteine residues (found in *C. reinhardtii* at amino acid positions 239 and 354) that have been implicated in colchicine binding in cross-linking studies of bovine brain  $\beta$ -tubulins (Little and Luduena, 1985). However, further phenotypic analysis of the mutants has suggested that the colchicine-resistant phenotypes of *col<sup>R</sup>4* and *col<sup>R</sup>15* are a consequence of an alteration in the assembly and stability of the microtubules, rather than a specific alteration in the drug-binding characteristics of the tubulin molecules per se (Schibler and Huang, 1987). Thus, lysine 350 appears to define a structural domain that through its juxtaposition in the overall conformation of the  $\beta$ 2-tubulin molecule influences the intrinsic assembly properties of the molecule and, hence, the ultimate stability of the assembled microtubules.

Indeed, we have recently obtained additional genetic evidence that the *col<sup>R</sup>* mutations at lysine 350 affect intrinsic tubulin-tubulin interactions. Through reversion analysis of *col<sup>R</sup>4*, an extragenic mutation has been isolated that not only suppresses the mutant drug sensitivity phenotypes of both *col<sup>R</sup>4* and *col<sup>R</sup>15*, but also confers in a wild-type background a pattern of altered sensitivity to microtubule inhibitors complementary to that of the  $\beta$ 2-tubulin mutations, i.e., supersensitivity to colchicine and other microtubule-disrupting drugs (Schibler and Huang, 1989). Recombination analysis indicates that this reciprocal suppressor mutation maps to the left arm of linkage group IV. The  $\alpha$ 2-tubulin locus in *C. reinhardtii*, as determined by restriction fragment length polymorphism, has been mapped to this same region (Ranum et al., 1988).

In our previous studies, we observed that the colchicine-resistant phenotypes of the mutants were expressed in the germination of heterozygous mutant zygotes and that both wild-type  $\beta$ 1-tubulins and mutant  $\beta$ 2-tubulins were coassembled into the flagellar microtubules in the haploid mutant strains (Bolduc et al., 1988). The observed codominant effect of the *col<sup>R</sup>* mutations, coupled with the observation that lysine 350 is a conserved residue in all the higher plant and animal  $\beta$ -tubulins sequenced to date, suggests that the codon for lysine 350 could be a good candidate for in vitro mutagenesis and gene replacement analysis in other systems for the further study of the effect of alteration in the tubulin molecule on the cellular functions and specificities of microtubules. Experiments of this kind may be of particular significance in higher plant systems because the *col<sup>R</sup>* mutations confer an increased resistance to the growth-inhibitory effects of a number of different herbicides (Schibler and Huang, 1987).

Although we have demonstrated only at the biochemical level that the variant  $\beta$ -tubulins are incorporated into the microtubules of the flagellum, the observation that the mutations confer an increased resistance to the inhibitory

effects of colchicine on vegetative cell division, on germination of meiotic products, and on flagellar assembly suggests that the  $\beta$ 2-tubulin in the mutants and, therefore, the  $\beta$ 2-tubulin in wild-type cells is very likely assembled into microtubules that participate in mitotic and meiotic cell divisions, in addition to flagellar formation. It remains to be determined whether the  $\beta$ 1-tubulin gene product is also involved in multiple microtubule functions. A detailed analysis of the expression of the two  $\beta$ -tubulin genes from transcription to assembly of their encoded proteins in *Chlamydomonas* is of particular interest because the products of the two  $\beta$ -tubulin genes are identical. If the genes are found to be differentially expressed during the cell cycle, the question will arise as to how and why the sequences of the gene products have been so rigidly conserved. On the other hand, if the genes are found to be coordinately expressed throughout the cell cycle, the functional significance of maintaining duplicate  $\beta$ -tubulin genes will need to be addressed.

The data presented in this report identify the genetic locus defined by the *col<sup>R</sup>4* and *col<sup>R</sup>15* mutations and previously mapped to the right arm on linkage group XII (Bolduc et al., 1988) as the site of the structural gene for the  $\beta$ 2-tubulin in *C. reinhardtii*. In accordance with the nomenclature that has been used to designate the tubulin genes in higher plant systems (Silflow et al., 1987), we suggest that the locus be designated *TUB2*, reserving the designation of *TUB1* for the  $\beta$ 1-tubulin gene locus. Recent results from restriction fragment length polymorphism mapping have provided evidence that the two  $\beta$ -tubulin genes in *C. reinhardtii* are distantly linked on the same chromosome (Ranum et al., 1988). Taken together, the data suggest that the structural gene for  $\beta$ 1-tubulin also resides on linkage group XII with a more distal location on the right arm.

## METHODS

### Construction of cDNA Libraries and Isolation of cDNA Clones

Poly(A)<sup>+</sup> RNA was isolated from the mutants 40 min after deflagellation as described previously (Bolduc et al., 1988) and was used as a template for cDNA synthesis with a commercially available cDNA synthesis system (Amersham Corp.) according to the manufacturer's specification, which was modified from the method of Gubler and Hoffman (1983). After ligation to  $\lambda$ gt11 phage arms, the cDNA was then packaged in vitro (Gigapack Plus, Stratagene Cloning Systems).

After amplification, the cDNA libraries were propagated in *Escherichia coli* Y1090 and screened with a <sup>32</sup>P-labeled probe by plaque hybridization. The probe for selection of  $\beta$ 2-tubulin clones was pcf8-31 (Schloss et al., 1984; Youngblom et al., 1984), a full-length  $\beta$ 2-tubulin cDNA inserted into the plasmid vector pBR322.

Phages that gave positive signal were plaque purified, their DNA was prepared, and the cDNA inserts were excised with EcoRI and subcloned by standard methods.

### Hybridization Selection

Linearized plasmid DNA (10- $\mu$ g samples) was denatured in 0.24 N NaOH, neutralized with 1 M ammonium acetate, and transferred to nitrocellulose (Schleicher & Schuell) by slow filtration. The filter was washed first with 1 M ammonium acetate and then with  $4 \times$  SSC ( $1 \times$  SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), air dried, and then baked at 80°C under vacuum for 2 hr. The portions of nitrocellulose that contained bound DNA were excised with a sterile paper punch. Prehybridization and hybridization were done in 50% (v/v) deionized formamide, 20 mM Pipes at pH 6.4, 0.4 M NaCl, 0.2% NaDodSO<sub>4</sub>, and yeast tRNA (Sigma Chemical Co.) at 100  $\mu$ g/mL. Prehybridization was for 1 hr at 41°C, and hybridization with 5  $\mu$ g of poly(A)<sup>+</sup> RNA per filter disc was for 2 hr at 41°C in 100- $\mu$ L aliquots. The filter discs were washed extensively under one of two conditions: (1) 10 mM Tris at pH 7.6, 150 mM NaCl, 1 mM EDTA, and 0.5% NaDodSO<sub>4</sub> at 55°C, or (2) 5 mM sodium phosphate at pH 7.7, 90 mM NaCl, 0.5 mM EDTA, and 0.5% NaDodSO<sub>4</sub> at 65°C. Filters were washed with the same wash solutions in the absence of NaDodSO<sub>4</sub> before elution.

For elution of RNA from the hybrid, filter discs were boiled for 1 min in 300  $\mu$ L of H<sub>2</sub>O containing 40  $\mu$ g of yeast tRNA, frozen quickly in a dry ice-ethanol bath, and thawed at room temperature. The eluted RNA was phenol extracted and then precipitated in 0.3 M sodium acetate and 70% ethanol. The recovered RNA was used for translation *in vitro* (see below).

### In Vitro Transcription and Translation

The EcoRI-digested inserts from the  $\lambda$ gt11 phage vector that contained the mutant  $\beta$ 2-tubulin cDNAs were inserted into the pT7/T3-18 vector (Bethesda Research Laboratories) at the EcoRI site by standard procedures. Similarly, the XhoI-BamHI fragment from pcf8-31, which contained the full-length cDNA coding region for the wild-type  $\beta$ 2-tubulin and more than 50 bp of both the 5'-untranslated and 3'-untranslated regions, was inserted into the pT7/T3-19 vector (Bethesda Research Laboratories) at the Sall and BamHI sites, respectively. The cohesive ends produced by Sall and XhoI digestion were complementary, although the ligation destroyed both endonuclease recognition sites at the Sall-XhoI junction. Coding (sense) RNA was synthesized by cleaving the pT7/T3-18 vector with HindIII and the pT7/T3-19 vector with EcoRI and performing *in vitro* transcription with T7 RNA polymerase (Bethesda Research Laboratories).

*In vitro* translation of the RNA synthesized off the transcription vectors was performed by using a commercially available rabbit reticulocyte lysate (Amersham Corp.) with added <sup>35</sup>S-methionine (Amersham Corp.). Products of *in vitro* translation were resolved in one-dimensional IEF gels as described previously (Bolduc et al., 1988).

### DNA Sequencing

cDNA inserts excised from the  $\lambda$ gt11 phage vector were digested with endonucleases to generate smaller fragments that were then subcloned into the M13mp18 and M13mp19 vectors using standard methods. Inserts were sequenced using the 17-mer universal primer for the Klenow fragment of DNA polymerase by the dideoxy

chain-termination method (Sanger et al., 1977; Biggin et al., 1983). Some regions were sequenced using synthetic oligonucleotides prepared against previously sequenced segments as primers. Both strands of the inserts were completely sequenced. DNA sequences were compiled and analyzed using PC Gene software (Intelligenetics, Palo Alto, CA).

### ACKNOWLEDGMENTS

We thank Dr. Carolyn Silflow for providing the plasmids pcf8-31 and pcf9-12 used in this study. This work was supported by Grant GM-38113 from the National Institutes of Health to B.H. V.D.L. was supported in part by an American Cancer Society postdoctoral fellowship.

Received June 25, 1990; accepted August 20, 1990.

### REFERENCES

- Adams, G.M.W., Huang, B., Piperno, G., and Luck, D.J.L. (1981). Central-pair microtubular complex of *Chlamydomonas flagella*: Polypeptide composition as revealed by analysis of mutants. *J. Cell Biol.* **91**, 69–76.
- Biggin, M.D., Gibson, T.J., and Hong, G.F. (1983). Buffer gradient gels and <sup>35</sup>S label as an aid to rapid DNA sequence determination. *Proc. Natl. Acad. Sci. USA* **80**, 3963–3965.
- Bolduc, C., Lee, V.D., and Huang, B. (1988).  $\beta$ -Tubulin mutants of the unicellular green alga *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. USA* **85**, 131–135.
- Cabral, F., Schibler, M., Kuriyama, R., Abraham, I., Whitfield, C., McClurkin, C., Mackensen, S., and Gottesman, M.M. (1984). Genetic analysis of microtubule function in CHO cells. In *Molecular Biology of the Cytoskeleton*, G.G. Borisy, D.W. Cleveland, and D.B. Murphy, eds (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory), pp. 305–317.
- Cleveland, D.W., and Sullivan, K.F. (1985). Molecular biology and genetics of tubulin. *Annu. Rev. Biochem.* **54**, 331–365.
- Dustin, P. (1984). *Microtubules*, 2nd ed (New York: Springer-Verlag), pp. 1–452.
- Fridovich-Keil, J.L., Bond, J.F., and Solomon, F. (1987). Domains of  $\beta$ -tubulin essential for conserved functions *in vivo*. *Mol. Cell. Biol.* **7**, 3792–3798.
- Fuller, M.T., Caulton, J.H., Hutchens, J.A., Kaufman, T.C., and Raff, E.C. (1988). Mutations that encode partially functional  $\beta$ 2-tubulin subunits have different effects on structurally different microtubule arrays. *J. Cell Biol.* **107**, 141–152.
- Gubler, U., and Hoffman, B.J. (1983). A simple and very efficient method for generating cDNA libraries. *Gene* **25**, 263–269.
- Gunning, B.E.S., and Hardham, A.R. (1982). Microtubules. *Annu. Rev. Plant Physiol.* **33**, 651–698.
- Huffaker, T.C., Thomas, J.H., and Botstein, D. (1988). Diverse effects of  $\beta$ -tubulin mutations on microtubule formation and function. *J. Cell Biol.* **106**, 1997–2010.

- Jung, M.K., Dunne, P.W., Suen, I.-H., and Oakley, B.R.** (1987). Sequence alterations in  $\beta$ -tubulin mutations of *Aspergillus nidulans*. *J. Cell Biol.* **105**, 277a (abstr).
- Katz, W.S., and Solomon, F.** (1988). Diversity among  $\beta$ -tubulins: A carboxy-terminal domain of yeast  $\beta$ -tubulin is not essential in vivo. *Mol. Cell. Biol.* **8**, 2730–2736.
- Lefebvre, P.A., Silflow, C.D., Wieben, E.D., and Rosenbaum, J.L.** (1980). Increased levels of mRNAs for tubulin and other flagellar proteins after amputation or shortening of *Chlamydomonas* flagella. *Cell* **20**, 469–477.
- Little, M., and Luduena, R.F.** (1985). Structural differences between brain  $\beta$ 1- and  $\beta$ 2-tubulins: Implications for microtubule assembly and colchicine binding. *EMBO J.* **4**, 51–56.
- Matthews, K.A., and Kaufman, T.C.** (1987). Developmental consequences of mutations in the 84B  $\alpha$ -tubulin gene of *Drosophila melanogaster*. *Dev. Biol.* **119**, 100–114.
- May, G.S., Gambino, J., Weatherbee, J.A., and Morris, N.R.** (1985). Identification and functional analysis of beta-tubulin genes by site specific integrative transformation in *Aspergillus nidulans*. *J. Cell Biol.* **101**, 712–719.
- Morejohn, L.C., and Fosket, D.E.** (1986). Tubulins from plants, fungi, and protists. In *Cell and Molecular Biology of the Cytoskeleton*, J.W. Shay, ed (New York: Plenum Press), pp. 256–329.
- Oakley, B.R.** (1985). Microtubule mutants. *Can. J. Biochem. Cell Biol.* **63**, 479–488.
- Orbach, M.J., Porro, E.B., and Yanofsky, C.** (1986). Cloning and characterization of the gene for  $\beta$ -tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as a dominant selectable marker. *Mol. Cell. Biol.* **6**, 2452–2461.
- Raff, E.C.** (1984). Genetics of microtubule systems. *J. Cell Biol.* **99**, 1–10.
- Ranum, L.P.W., Thompson, M.D., Schloss, J.A., Lefebvre, P.A., and Silflow, C.D.** (1988). Mapping flagellar genes in *Chlamydomonas* using restriction fragment length polymorphisms. *Genetics* **120**, 109–122.
- Sanger, F., Nicklen, S., and Coulson, A.R.** (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
- Schatz, P.J., Georges, G.E., Solomon, F., and Botstein, D.** (1987). Insertions of up to 17 amino acids into a region of  $\alpha$ -tubulin do not disrupt function in vivo. *Mol. Cell. Biol.* **7**, 3799–3805.
- Schibler, M.J., and Huang, B.** (1987). Effects of herbicides and microtubule inhibitors on  $\beta$ -tubulin mutants of *Chlamydomonas reinhardtii*. *J. Cell Biol.* **105**, 29a (abstr).
- Schibler, M.J., and Huang, B.** (1989). An extragenic suppressor of the *col<sup>R</sup>4*  $\beta$ -tubulin mutant of *Chlamydomonas reinhardtii* maps to the  $\alpha$ 2-tubulin locus. *J. Cell Biol.* **109**, 339a (abstr).
- Schloss, J.A., Silflow, C.D., and Rosenbaum, J.L.** (1984). mRNA abundance changes during flagellar regeneration in *Chlamydomonas reinhardtii*. *Mol. Cell. Biol.* **4**, 424–434.
- Silflow, C.D., and Rosenbaum, J.L.** (1981). Multiple  $\alpha$ - and  $\beta$ -tubulin genes in *Chlamydomonas* and regulation of tubulin mRNA levels after deflagellation. *Cell* **24**, 81–88.
- Silflow, C.D., Oppenheimer, D.G., Kopczak, S.D., Ploense, S.E., Ludwig, S.R., Haas, N., and Snustad, D.P.** (1987). Plant tubulin genes: Structure and differential expression during development. *Dev. Genet.* **8**, 435–460.
- Sullivan, K.F.** (1988). Structure and utilization of tubulin isotypes. *Annu. Rev. Cell Biol.* **4**, 687–716.
- Sullivan, K.F., and Cleveland, D.W.** (1986). Identification of conserved isotype-defining variable region sequences for four vertebrate  $\beta$ -tubulin polypeptide classes. *Proc. Natl. Acad. Sci. USA* **83**, 4327–4331.
- Thomas, J.H., Neff, N.F., and Botstein, D.** (1985). Isolation and characterization of mutations in the  $\beta$ -tubulin gene of *Saccharomyces cerevisiae*. *Genetics* **112**, 715–734.
- Youngblom, J., Schloss, J.A., and Silflow, C.D.** (1984). The two  $\beta$ -tubulin genes of *Chlamydomonas reinhardtii* code for identical proteins. *Mol. Cell. Biol.* **4**, 2686–2696.