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Coordinate regulation of the four tubulin genes of Chlamydomonas reinhardi

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ABSTRACT

During cell division and during the induction of tubulin synthesis that accompanies flagellar regeneration in Chlamydomonas reinhardi, four tubulin mRNAs of discrete molecular sizes are produced. During induction two β tubulin mRNAs (2.47 kb and 2.34 kb) and two α tubulin mRNAs (2.26 kb and 2.13 kb) are synthesized in high abundance and in a closely coordinated fashion. Combined data from restriction enzyme mapping (i.e., Southern analysis) of genomic DNA and of Charon 30 recombinant clones bearing inserts of Chlamydomonas tubulin genes provide direct evidence for four distinct tubulin genes in this organism. Dot-blot analysis of the level of hybridization of a ^{32}P nick-translated β tubulin cDNA to genomic DNA from gametic cells and to a clone containing the β_1 tubulin gene indicate that each β_1 tubulin gene is present in one copy per cell. Additional hybridization experiments employing fragments of cDNA clones which selectively anneal to either the 3' or 5' portions of the two α tubulin genes or to one or both of the two β tubulin genes suggest that each tubulin gene is actively transcribed to give rise to one of the four tubulin mRNAs. These observations further suggest that at most four basic types of tubulin subunits are produced by Chlamydomonas and that the heterogeneity of tubulin subunits reported to exist in the flagellar axoneme must arise as a result of post-translational modification.

INTRODUCTION

The unicellular alga, Chlamydomonas reinhardi, possesses two flagella. In nature these flagella provide the organism with the motility needed to seek out the most favorable local environment in which to survive. They also play an integral role in the pairing of gametic cells of opposite mating type during the mating reaction (1). If flagella are lost, a new set of flagella is regenerated within 60 to 90 min. In the laboratory this phenomenon has been exploited to study the regeneration of a cellular organelle (2-4) and to examine the mechanisms involved in the controlled synthesis of individual flagellar proteins. Much attention has been focused on tubulin, the most abundant flagellar protein, since its synthesis has been shown to be rapidly induced to high levels soon after flagellar excision (5-8). Recent experiments in our laboratory have detected increases in tubulin mRNA synthesis

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in less than 5 min after deflagellation (6). These experiments using cDNA clones complementary to tubulin mRNA, and similar experiments by Silflow *et al.* (7), have shown that tubulin mRNA accumulation peaks between 30 and 60 min after flagellar excision and then declines to control levels over a 120 to 150 min period. The observed close coupling between tubulin subunit synthesis and tubulin mRNA concentrations (6) suggests that the synthesis and degradation of tubulin mRNA are the key factors in controlling tubulin production during flagellar regeneration.

Examination of tubulin from *C. reinhardi* (9, 10, 11) and other organisms (e.g., 12, 13, 14) has revealed multiple types of α and β tubulin subunits. Such microheterogeneity is not surprising in view of the diverse cellular functions in which microtubules are involved and the variety of macromolecules with which they interact (3, 15, 16). The presence of multiple genes for tubulin in several complex eukaryotes [for example, 8 in chicken (17), approximately 10-14 in humans (18) and as many as 20 in sea urchin (17)] provides an explanation for at least a portion of this variation in tubulin subtypes. However, it has been suggested that in *Chlamydomonas* there may be as few as four tubulin genes (6, 7) and that some tubulin subunit heterogeneity may arise as a result of post-translational modification (9, 10). We now provide definitive evidence for four distinct genes in *Chlamydomonas* based on coupled data from both Southern analysis of total genomic DNA and from restriction enzyme mapping of the four tubulin genes cloned into the bacteriophage, Charon 30. Additional results indicate that the four genes give rise to four distinct tubulin mRNAs that are synthesized in a highly coordinate fashion during flagellar regeneration. These data which suggest that at most four types of tubulin subunits are produced by translation in *Chlamydomonas* are discussed in regard to new evidence from our laboratory that the post-translational modification of certain flagellar tubulins may be dependent upon flagellar outgrowth.

MATERIALS AND METHODS

Strains and growth conditions. Cultures of *C. reinhardi* strain 137C+ or 137C- were maintained under conditions previously described (19) for synchronous vegetative growth or for gamete formation.

Isolation of mRNA and DNA. RNA and DNA were extracted from either vegetative or gametic cells by methods previously described (6) or by a modification of procedures by Wolf (20) and Gonzalez *et al.* (21). Gametic cells to be deflagellated were harvested, concentrated 10-fold, and then deflagellated by pH

shock (22). Except where indicated in figure legends, cells were allowed to regenerate flagella for 40 min, then quick-chilled on ice, and harvested by centrifugation. Pelleted cells were lysed in 2% Sarkosyl, 100 mM Tris-HCl pH 8.8, 10 mM EDTA, 100 mM NaCl and 0.4 mg/ml predigested Pronase as previously described (6). At this stage, one of two alternative procedures was followed: 1) Total nucleic acid was extracted with chloroform-phenol and ethanol precipitated (6). When needed, polyadenylated mRNA was obtained by fractionation of total nucleic acid on oligo (dT)-cellulose columns.
2) Digested lysates were combined with 1.75 vol of 5.8 M potassium iodide, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% Sarkosyl, and 20 μg/ml ethidium bromide. 22 ml of this mixture was layered over 16.3 ml of the 5.8 M potassium iodide buffer solution in a Beckman quick-seal tube. The layered samples were then centrifuged at 20° and 38,000 rpm for 40 hrs in a Ti 50 rotor. RNA and DNA bands were removed from the tube by side-puncture and extracted with isoamyl alcohol to remove ethidium bromide. DNA was precipitated in 1 vol of cold ethanol and spooled onto a glass rod while RNA was diluted with 3 vol of H_2O and precipitated in cold 70% ethanol. Both DNA and RNA precipitates were centrifuged and washed with absolute ethanol before resuspension in appropriate buffers.

Northern and Southern blot analysis. RNA separated in 1% agarose gels (44 cm long) containing 10 mM methylmercury hydroxide (23) was transferred to nitrocellulose by the method of Thomas (24). DNA digested with a 2- to 10-fold excess of each restriction enzyme (New England Biolabs or Bethesda Research Labs) for 6 to 18 hr was separated by electrophoresis in 0.7% agarose gels and transferred to nitrocellulose according to the procedures of Southern (25). ^{32}P-labeled DNA hybridization probes were prepared by the nick-translation methods of Rigby *et al.* (26). Hybridization procedures were those of Wahl *et al.* (27).

Analysis of *Chlamydomonas* tubulin gene restriction fragments containing 3' or 5' coding regions was performed using 3' and 5' specific probes of chicken α and β tubulin cDNAs as described by Cleveland *et al.* (17) and a 3'-specific cDNA probe for β tubulin mRNA from *Chlamydomonas*. Dot-blot analysis (28) was carried out using a Bethesda Research Laboratories Hybri-Dot apparatus.

Preparation and screening of C. reinhardi genomic DNA library. Construction of a library of *Chlamydomonas* genomic DNA and *in vitro* packaging of recombinant molecules were carried out by standard procedures (29). Genomic DNA was prepared by partial digestion with Sau 3A, sucrose gradient fractionation,

and insertion into the Bam HI site of Charon 30. The unamplified library was screened for tubulin genes by the methods of Benton and Davis (30) using chicken α and β tubulin cDNA probes (17). DNA was isolated either from plate lysates or from liquid cultures of plaque-purified phage (31). From an initial screening of approximately 150,000 plaques, 21 clones with an average insert size of ∿ 12 kb were chosen for restriction map analysis of the tubulin genes. For detailed restriction enzyme analysis, we used Charon clone 15 containing the β_1 tubulin gene, Charon clones 14 and 20 containing the β_2 gene, Charon clone 9 containing the α_1 gene, and Charon clone 27 containing the α_2 gene.

RESULTS

Coordinate induction of tubulin mRNAs. Previous studies (6, 7) have shown that the rapid induction of tubulin synthesis that follows flagellar excision is dependent on the increased synthesis of tubulin mRNAs. The production of new tubulin subunits during flagellar regeneration is closely tied to the concentration of tubulin mRNA during the induction-deinduction cycle (6). The data of Figure 1 extend these earlier findings by showing that four separate mRNAs are produced during flagellar regeneration as well as during cell division (Fig. 1A). The two largest mRNAs, designated β_1 and β_2, hybridize at high stringency to cDNA clones of chicken β tubulin mRNA (17) (Fig. 1A) and to clones of genomic DNA containing either of the two *Chlamydomonas* β tubulin genes (see below). The two smaller mRNAs, designated α_1 and α_2, are likewise identified as α tubulin mRNAs by their selective hybridization to α tubulin gene probes. The mRNAs produced during flagellar regeneration have identical electrophoretic mobilities as those produced during the period of intense tubulin synthesis that accompanies synchronous cell division (19) in vegetative cells (cf. lane 1 vs. lane 3, Fig. 1B). The size similarities suggest that the same four tubulin mRNAs are produced during both cell division and flagellar regeneration.

We can not rigorously rule out the possibility that any one of the four size classes of tubulin mRNA may contain more than one distinct molecular species. However, it may be noted that the cDNA plasmid, pFTβ1, which we described earlier (6) hybridizes strongly with only the largest of the two β tubulin mRNAs (Fig. 1B). The selective hybridization of pFTβ1 to only one of the two β tubulin mRNA species also suggests that the two mRNAs are most likely encoded by two separate genes. This possiblity is strengthened by the observation that when denatured pFTβ1 plasmid DNA is hybridized with an

excess of Chamydomonas mRNA from deflagellated cells and then subjected to S_1 nuclease digestion (32),the β_1 mRNA that hybridizes to the pFTβ1 DNA protects a fragment over 350 nucleotides in length (data not shown). Since the β_1 and β_2 tubulin mRNAs differ in size by only 130 nucleotides (Fig. 1A) and pFTβ1 DNA hybridizes to a sequence of at least 350 nucleotides in the β_1 tubulin mRNA, it is unlikely that the two mRNAs are encoded by a single gene and that β_2 mRNA is simply a shortened species of the β_1 mRNA.

The rise and fall in concentration of all four tubulin mRNAs during tubulin induction in deflagellated cells is highly coordinate (Fig. 1C and D). The four messengers appear to be synthesized in constant ratios to one another and in comparable quantities judging from the hybridization with either labeled cDNA clones (Fig. 1) or clones of the tubulin genes (not shown).

Estimation of the number of tubulin genes. Initial estimates of the number of genes in C. reinhardi coding for α and β tubulin were obtained by Southern analysis of genomic DNA digested by pairwise combinations of restriction enzymes with six base recognition (Bam HI, Sma I, Bst EII, and Xho I). The DNA restriction fragments resulting from these digests were separated by electrophoresis in a 0.7% agarose gel and transferred to a nitrocellulose filter. When the resulting filter was incubated with ^{32}P nick-translated pFTβ1, which selectively hybridizes to the largest of the β tubulin mRNAs (arbitrarily designated the β_1 tubulin mRNA), only one DNA fragment in each digest (Fig. 2A, lanes 1-4) was detected. However, when the same filter was probed with a 3'-specific probe derived from a cDNA clone of chicken β tubulin mRNA (17) (Fig. 2B), one additional band of radioactivity was detected in each digest. [The Bam HI-Sma I digest (lane 2) contains two fragments of nearly identical size].

Since the 3'-specific probe from chicken β tubulin cDNA hybridized to each of the Chlamydomonas β tubulin mRNAs, the fragments labeled 1 and 2 in Figure 2B appear to correspond to two separate β tubulin genes. This conclusion was borne out by further hybridizations of the filter with a portion of the chicken β tubulin cDNA that selectively hybridizes to the 5' portion of β tubulin messenger RNA (Fig. 2C). In the digest with the enzyme pair, Xho I and Bst EII (lane 4 of Fig. 2C), the same two fragments were hybridized with the 5' probe as were hybridized with the 3' probe. This suggests that the Xho I and Bst EII restriction sites are to either side of the coding regions in both gene β_1 and gene β_2. On the other hand, the appearance of a new fragment of the β_1 gene which hybridized to the 5'-specific probe in each of the

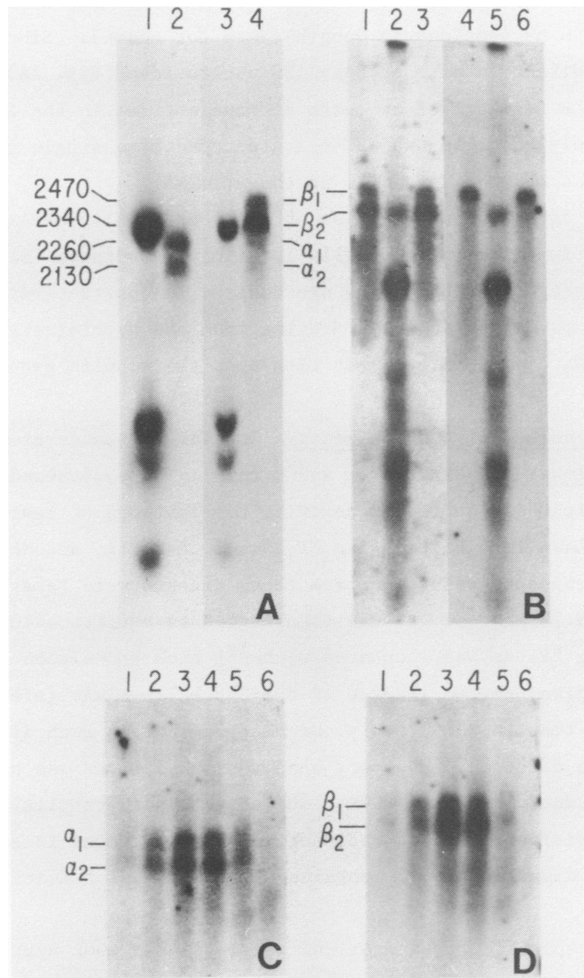

Figure 1. Tubulin mRNAs of C. reinhardi. Polyadenylated mRNA (A and B) or total cell nucleic acid (C and D) isolated from deflagellated gametic cells or from vegetative cells during cell division was denatured in 20 mM MeHgOH and separated by electrophoresis through 1% agarose gels containing 10 mM MeHgOH. After transfer to nitrocellulose filters the tubulin mRNAs were probed with ^{32}P nick-translated DNA from clones of either *Chlamydomonas* β tubulin cDNA or chicken α or β tubulin cDNAs.

A) Molecular sizes of *Chlamydomonas* tubulin mRNAs. Polyadenylated mRNA (20 μg/lane) isolated from synchronized vegetative cells during cell division and probed with cloned chicken cDNA complementary to α tubulin mRNA (lane 2) or β tubulin mRNA (lane 4). Lanes 1 and 3 contain size markers from Pvu II and Hinc II double digest of pFTβ2 (6) with lengths (in nucleotides) of 2393, 1415, 1302, and 1060.

B) Selective hybridization of the Chlamydomonas cDNA plasmid pFTβ1 to β_1 tubulin mRNA. Polyadenylated mRNA (2 μg of mRNA fractionated once through oligo dT-cellulose) from deflagellated gametic cells (lanes 1 and 4) or from dividing vegetative cells (lanes 3 and 6; 2 μg of mRNA fractionated 3 times through oligo dT-cellulose) hybridized with cloned chicken β tubulin cDNA (lanes 1-3) or Chlamydomonas β tubulin cDNA clone, pFTβ1 (lanes 4-6). Lanes 2 and 5 contain a mixture of restriction enzyme digests as molecular size markers (Bst NI digest of pBR322: 1857, 1060, 928; Pvu II-Hinc II double digests of pFTβ2: 2393, 1415, 1302, 1060; Eco RI-Pst I double digests of pFTβ2: 3730, 1415, 1290, 730).

C and D) Induction of tubulin mRNA synthesis following flagellar excision. Total cell nucleic acid (20 μg/lane) isolated from control (lane 1) or deflagellated cells at 15, 30, 60, 90 or 180 min after flagellar excision (lanes 2, 3, 4, 5 and 6, respectively) and probed with ^{32}P nick-translated DNA from plasmids containing chicken α tubulin cDNA (C) or β tubulin cDNA (D) sequences.

digests containing Bam HI (lanes 1-3, Fig. 2C) demonstrates that the β_1 gene, but not the β_2 gene, contains an internal Bam HI cleavage site that divides the gene into 3' and 5' portions. If these data concerning the β_1 gene are coupled with the fact that the 2100 bp fragment of the β_2 gene extending from the Bam HI site to the Xho I site is only large enough to contain one complete β tubulin gene sequence, then the only conclusion consistent with all of the data presented here is that there are only two distinct genes which code for β tubulin in Chlamydomonas. Moreover, data from the dot-blot analysis (28) shown in Figure 3 suggest that there is only one copy of the β_1 tubulin gene in the haploid genome of Chlamydomonas. (This represents one gene per cell since in Chlamydomonas both vegetative and gametic cells are haploid.) In this analysis, the ^{32}P nick-translated cDNA plasmid, pFTβ1, which hybridizes selectively to the β_1 tubulin gene (Fig. 2) was hybridized to a quantity of genomic DNA that contains, for example, 10^7 copies of the Chlamydomonas genome (row 2, spot 5). The intensity of hybridization as judged by visual comparisons and densitometer analyses of several different exposures of the autoradiograph was the same as the intensity of hybridization to a quantity of β_1 gene Charon clone 15 that represents 10^7 copies of the phage DNA (row 1, spot 5).

To insure that the chicken β tubulin cDNA clone used in some of our experiments detected all of the β tubulin genes in Chlamydomonas, we also carried out a Southern analysis of genomic DNA cleaved with a variety of enzymes and probed with the 2.1 kb fragment from the Bam HI site to the Xho I site of a cloned β_2 tubulin gene which contains all or nearly all of the coding region (see Fig. 5). This homologous probe detected the same restriction fragments as the chicken β tubulin cDNA probe and no others (Fig. 4A).

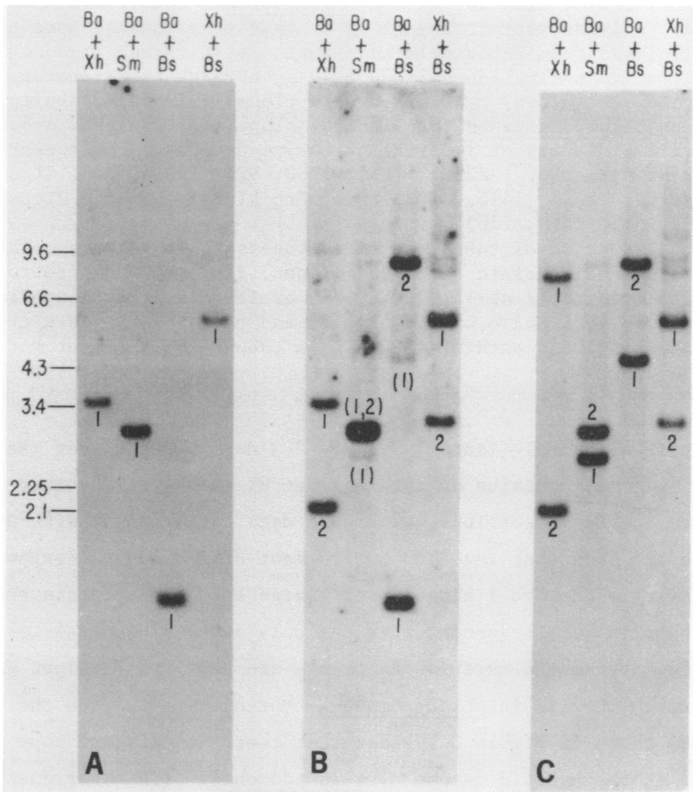

Figure 2. Restriction endonuclease-digested genomic DNA hybridized with cDNA probes complementary to the 5' and 3' ends of β tubulin mRNA. Fragments from the digestion of genomic DNA with combinations of Bam HI (Ba), Xho I (Xh), Sma I (Sm), and Bst EII (Bs) were separated by electrophoresis through a 0.7% agarose gel, blotted onto nitrocellulose, and then hybridized with ^{32}P nick-translated probes. A] Genomic DNA restriction fragments probed with pFTβ1, a Chlamydomonas cDNA clone containing 3' sequences complementary to β_1 tubulin mRNA; B] Fragments probed with portions of chicken β tubulin cDNA that contains sequences complementary to the 3' portion of β_1 and β_2 tubulin mRNAs; C] Restriction fragments probed with a portion of chicken β tubulin cDNA that contains sequences complementary to the 5' portion of β_1 and β_2 tubulin mRNAs. The left margin gives molecular sizes (in kb) for some Hind III and Eco RI restriction fragments of λ DNA which were used as size markers (detected by ethidium bromide staining). Each fragment has been designated by its hybridization characteristics as being a portion of the β_1 or β_2 tubulin gene. In Figure 2B, the band labelled (1, 2) is a doublet of two fragments, one fragment containing sequences from the β_1 tubulin gene and the other from the β_2 tubulin gene. The low amount of label in the two bands marked (1) indicates that these fragments may have a small region with limited homology to the 3' probe. All other minor bands can be accounted for as partial digestion fragments of the β_1 and β_2 tubulin genes (see Figure 5).

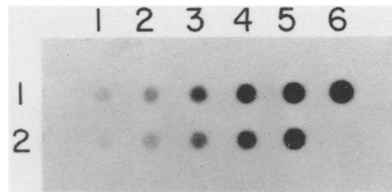

Figure 3. Quantitation of the number of β_1 genes in the haploid Chlamydomonas genome by dot-blot analysis. Chlamydomonas DNA and Charon clone number 15 containing the β_1 tubulin gene were digested with Bam HI, denatured, and applied along with 2 μg of bacteriophage PBS2 DNA per spot to a nitrocellulose filter held in a BRL hybri-dot apparatus. Spots 1 through 6 of row 1 contain 37, 62.5, 125, 250, 500, and 1000 pg of the 49 kb β_1 Charon clone. The 500 pg load size corresponds to 10^7 copies of the phage DNA. Spots 1 through 5 in row 2 of the filter contain 75, 150, 300, 600 and 1200 ng of Chlamydomonas DNA, respectively. The 1200 ng load size corresponds to 10^7 copies of the Chlamydomonas haploid genome. Spot number 6 of row 2 is a control containing only the 2 μg of carrier phage DNA. The DNA on the filter was hybridized with ^{32}P nick-translated cDNA plasmid pFTβ1 which selectively hybridizes to the 3' region of the β_1 tubulin gene. Densitometer scans of several exposures of the radioactive filter indicate that hybridization to each spot of Chlamydomonas DNA is between 76% and 86% as intense as to the spot of clone number 15 DNA containing an equivalent number of genomes (e.g., spot 5, row 1 vs. spot 5, row 2, etc.).

Interestingly, the ^{32}P-labeled 2.1 kb fragment appears to anneal slightly better with restriction enzyme fragments of its parent β_2 gene than to fragments of the β_1 gene. This could reflect a difference in hybridization due to the lack of homology in the non-coding regions of the two genes (e.g., see Fig. 2) and perhaps, to some extent, a divergence of sequences within the coding regions of the gene.

Southern analysis of restriction enzyme fragments of the Chlamydomonas tubulin genes using intact α tubulin cDNA probes (Fig. 4B) or probes specific for the 5' and 3' portions of the α tubulin genes (data not shown) again suggests, as in the case of the β tubulin genes, that only two distinct genes in C. reinhardi code for α tubulin.

Restriction endonuclease maps of the tubulin genes. To insure that our interpretation of the Southern analyses was correct and to gather more detailed information on the structure of the tubulin genes in Chlamydomonas, we prepared a Charon 30 library of genomic DNA and isolated independent clones bearing sequences complementary to a mixture of α and β tubulin cDNA clones. Out of the first 21 plaque-purified clones selected, four proved to be clones of the α tubulin genes and 17 were clones of the β tubulin genes. Pst I restriction enzyme analysis revealed two distinct families within each

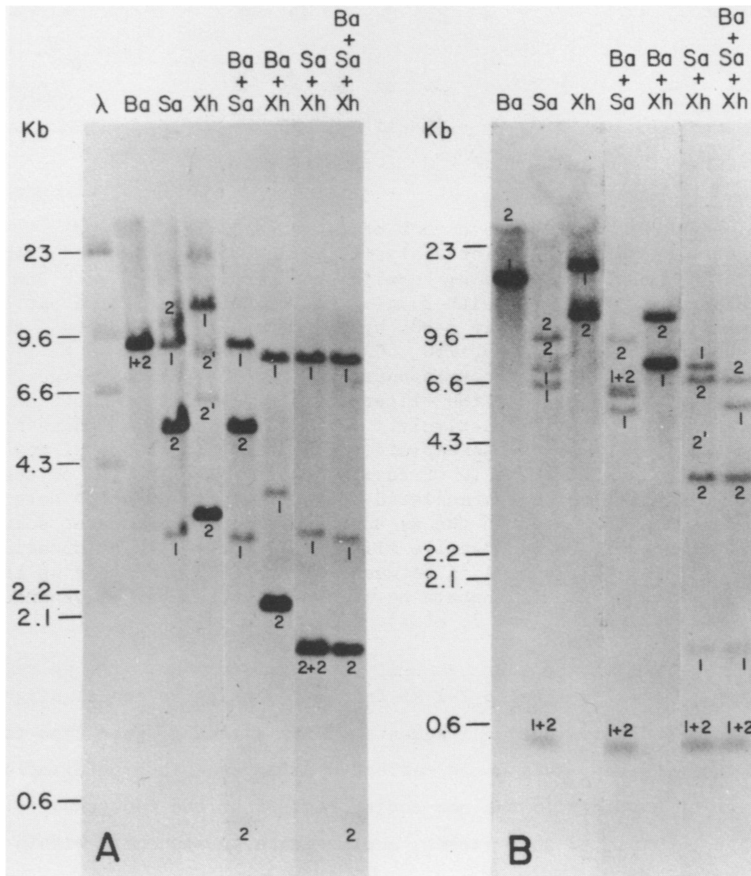

Figure 4. Southern analysis of the β tubulin genes (A) and α tubulin genes (B) of Chlamydomonas DNA. Genomic DNA was digested with Bam HI (Ba), Sal I (Sa), or Xho I (Xh) or combinations of these enzymes. Electrophoretic conditions and DNA transfer to nitrocellulose sheets are the same as those described in Figure 2. A) Autoradiograph of restriction enzyme fragments of the Chlamydomonas β_1 (designated with the number 1 above or below the respective fragment) or β_2 (designated with the number 2) tubulin genes probed with the ^{32}P nick-translated 2.1 kb fragment from the Bam HI to Xho I sites in the coding region of the β_2 gene (see Figure 5). Lane 1 contains a radioactively labelled Hind III digest of the bacteriophage λ. Labelled bands corresponding to partial digests of the genes are denoted by numbers with prime marks (see the restriction maps of Figure 5). B) Autoradiograph of restriction enzyme fragments of the Chlamydomonas α_1 (designated with the number 1) or α_2 (designated with the number 2) tubulin genes probed with ^{32}P nick-translated α tubulin cDNA from chicken. Again numbers with prime marks denote partially digested gene fragments. The number designations of the two α genes (i.e. α_1 and α_2) have been made arbitrarily and may or may not correspond to the number designations of the two α tubulin mRNAs.

set of α and β tubulin gene clones. One or more clones from each family were then subjected to restriction enzyme analyses with a variety of enzymes having six base pair recognition. The resulting restriction maps were completely compatible with all of the Southern analyses of genomic DNA that we had previously performed (the data of Fig. 2 and Fig. 4 being examples of such analyses). The subset of restriction fragments within the four cloned genes which hybridized with cloned Chlamydomonas or chicken tubulin cDNAs (i.e., transcribed regions) were the only fragments that were detected by Southern analyses of restriction enzyme digests of total genomic DNA -- indicating that the genes cloned into Charon 30 represented all of the tubulin genes in Chlamydomonas. Restriction enzyme maps of the four tubulin genes based on a combination of restriction enzyme analyses of both genomic DNA and the cloned genes are presented in Figure 5. While the restriction maps of the β_1 and β_2 genes show little similarity, the restriction sites within the transcribed regions of the α_1 and α_2 genes show marked similarities which suggest the possibility of gene duplication during recent evolutionary times. Nevertheless, the distances between the Sal I restriction sites for the two α tubulin genes are slightly, but distinctly, different (i.e., the two largest Sal I fragments in the Sal I cluster of the α_1 gene are approximately 500 bp and 140 bp while those for the α_2 gene are approximately 530 bp and 180 bp). Finally, we conclude that the four tubulin genes are not tightly linked since the restriction enzyme mapping data dictate that the minimum spacing between any two of the tubulin genes must be greater than ten kilobases.

DISCUSSION

The results of Southern analyses of genomic DNA (Figs. 2 and 4) as well as the characterization of cloned tubulin genes (Fig. 5) provide firm evidence for only four tubulin genes in Chlamydomonas — two genes encoding information for the α tubulin subunits and two encoding information for the β tubulin subunits. The presence of two size classes of α tubulin mRNA and two size classes of β tubulin mRNA in cells producing tubulin either during cell division or flagellar regeneration suggests that all four tubulin genes are expressed whenever tubulin synthesis is required by the cell. This suggestion is further strengthened by the observation that in the case of the β tubulin genes the 3'-specific cDNA clone, pFTβ1, hybridizes to only the larger species of β tubulin mRNA (Fig. 1B) and to only one of the β tubulin genes (Fig. 2). We have arbitrarily designated this gene as the β_1 tubulin gene of

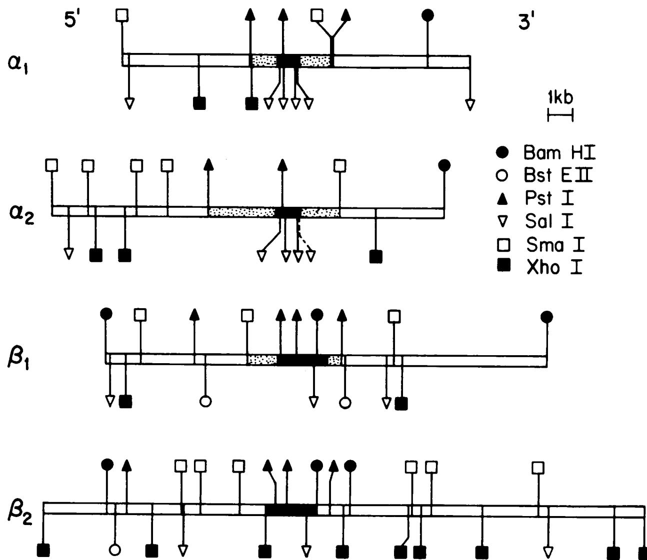

Figure 5. Restriction maps of α and β tubulin genes from Chlamydomonas. Southern analyses of restriction fragments from total genomic DNA probed with ^{32}P-labelled cDNA clones of tubulin mRNAs and restriction analyses of genomic DNA clones containing α and β tubulin genes (see Materials and Methods for details) were combined to construct the restriction maps above. Our knowledge concerning cutting sites for the individual restriction enzymes employed does not extend beyond the most leftward site and the most rightward site depicted for each enzyme in the four maps. Solid boxes denote known transcribed regions for the tubulin genes. For the β_2 tubulin gene, the size of the mRNA dictates that most of the DNA in the 2.1 kb fragment from the Bam HI to Xho I sites (solid box) is transcribed. The specific 5' and 3' limits of the transcribed regions for the remaining tubulin genes, are known to be within the restriction fragments indicated by the stippled areas. In the α_2 tubulin gene, a Sal I site for which the data is still tentative is represented by a vertical dashed line.

Chlamydomonas.

The synchronous appearance and disappearance of all four tubulin mRNAs (Fig. 1C and D) during the extensive induction of tubulin synthesis that follows flagellar excision (6) indicate a closely coordinated regulation of tubulin gene transcription and/or tubulin mRNA processing. These observations and similar observations on partially resolved tubulin mRNAs by Silflow et al. (7) suggest that the intracellular signals generated as a result of flagellar detachment bring about molecular changes that uniformly affect the production of mRNA from each of the tubulin genes. Whether the four genes carry common regulatory sequences is not known. However, it may be noted

that there is a marked similarity in the restriction enzyme maps of the two α tubulin genes. This similarity is especially evident in a comparison of the Sal I restriction sites clustered near the center of the coding region of the two α genes. These similarities indicate that the two α genes may have arisen as a result of gene duplication and, if so, could well share common regulatory sequences. Similarities between the β tubulin genes are less definite. Only a more detailed analysis of gene structure will allow us to determine whether the four tubulin genes in Chlamydomonas react with the same or similar "control" factors by virtue of having common nucleotide tracts.

There is a high degree of conservation of amino acid sequences within α or β subunits of tubulin isolated from a number of diverse organisms (33-36). The conservation of structure extends to the level of nucleotide sequence as evidenced by comparisons of tubulin genes from different organisms (35, 37) and is reflected in the strong cross-hybridization of tubulin cDNA clones with mRNAs of widely divergent origins (17). In the studies presented here, we have detected no tubulin gene sequences with ^{32}P nick-translated probes of cloned homologous Chlamydomonas tubulin genes that were not also detected with labelled chicken tubulin cDNA probes. Moreover, our estimates of tubulin gene number are in direct agreement with the initial estimates of Silflow, et al. (7) who used homologous tubulin cDNA probes from Chlamydomonas. (It nevertheless remains a tacit assumption in this study, and the studies of others, that there are no tubulin genes in the genome whose coding regions have diverged so widely that they are no longer detectable by cross-hybridization with tubulin cDNA or genomic probes presently available.) The strong cross-hybridization between sequences from the two members of the α or β gene families in Chlamydomonas may be confined to the coding regions of the gene, since at least one cDNA clone, pFTβ1, carries sequences complementary to the 3' non-translated region of one β tubulin mRNA but not the other (Fig. 1). Analogous observations have been made in regard to conserved and non-conserved nucleotide sequences within the actin multigene families of various organisms (38, 39).

Two observations suggest that there is only one copy of each of the four tubulin genes in the Chlamydomonas genome. First, if quantitation of the number of β_1 tubulin genes is made by dot-blot analysis (Fig. 4) using the cloned β_1 gene as a standard of comparison and ^{32}P nick-translated pFTβ1 as a probe that is specific for the β_1 gene, the results suggest that there is only one copy of the gene in each haploid genome. Secondly, our restriction maps for each of the four distinct tubulin genes extend over long distances

(from 13 kb for the α_1 gene to 23 kb for the β_2 gene). If there were duplicates of any or all of the tubulin genes in the genome, the repeat unit would have to extend over the entire distance we have mapped since we have found no evidence for sequence divergence in any of the four genes using a wide variety of restriction enzymes.

The low tubulin gene number in Chlamydomonas suggests that the diversity of tubulin subtypes seen within this organism may not depend solely on genetic variation. If the four tubulin genes in C. reinhardi (Fig. 5) give rise to only four different tubulin mRNAs (Fig. 1), then the existence of more than four types of tubulin subunits (2α and 2β) can only be explained by post-translational modification of the basic subunit types. Early work by Piperno and Luck (9) provided suggestive evidence that phosphorylation may be one form of post-translational modification of tubulin in Chlamydomonas. Differences in the electrophoretic mobilities of α tubulin subunits produced by translation of tubulin mRNAs in vitro compared with the major α tubulin subunits in the flagellum were interpreted by Lefebvre and co-workers (10) as evidence in favor of post-translational modification of α tubulin subunits. Recent experiments in our laboratory have confirmed this conclusion and have further demonstrated that the modification of α tubulin subunits in the flagellum occurs only during periods of active flagellar assembly. The apparent restriction of this particular type of α tubulin subunit modification to the flagellum suggests that it may have a role in promoting the specialized interactions of other flagellar proteins with the microtubules of the flagellar axoneme. In any case, it is apparent from the studies presented here that the multiplicity of interactions and functions which microtubules have in the Chlamydomonas flagellum and cell body are achieved with a limited set of not more than four tubulin subunits and that specialization of function for particular microtubules in different subcellular structures may be dependent on specific modes of post-translational modification.

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