

# Acetylation of Lysine 40 in $\alpha$ -tubulin Is Not Essential in *Tetrahymena thermophila*

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**Abstract.** In *Tetrahymena*, at least 17 distinct microtubule structures are assembled from a single primary sequence type of  $\alpha$ - and  $\beta$ -tubulin heterodimer, precluding distinctions among microtubular systems based on tubulin primary sequence isotypes. *Tetrahymena* tubulins also are modified by several types of posttranslational reactions including acetylation of  $\alpha$ -tubulin at lysine 40, a modification found in most eukaryotes. In *Tetrahymena*, axonemal  $\alpha$ -tubulin and numerous other microtubules are acetylated. We completely replaced the single type of  $\alpha$ -tubulin gene in the macronucleus with a version encoding arginine instead of lysine 40 and therefore cannot be acetylated at this position. No

acetylated tubulin was detectable in these transformants using a monoclonal antibody specific for acetylated lysine 40. Surprisingly, mutants lacking detectable acetylated tubulin are indistinguishable from wild-type cells. Thus, acetylation of  $\alpha$ -tubulin at lysine 40 is non-essential in *Tetrahymena*. In addition, isoelectric focusing gel analysis of axonemal tubulin from cells unable to acetylate  $\alpha$ -tubulin leads us to conclude that: (a) most or all ciliary  $\alpha$ -tubulin is acetylated, (b) other lysines cannot be acetylated to compensate for loss of acetylation at lysine 40, and (c) acetylated  $\alpha$ -tubulin molecules in wild-type cells contain one or more additional charge-altering modifications.

**M**ICROTUBULES are involved in cytoplasmic organization and motility in eukaryotic cells. They form a large variety of distinct organelles including a cytoplasmic network, the mitotic spindle, the centrosome, cilia, and flagella. The core component of microtubules is a heterodimer of  $\alpha$ - and  $\beta$ -tubulin proteins. In most organisms tubulins occur in multiple isoforms.<sup>1</sup> In lower eukaryotes such as *Saccharomyces cerevisiae*, *Aspergillus nidulans*, *Dictyostelium discoideum* and *Tetrahymena*, a relatively low number of tubulin isogenes is mainly involved in coordinate regulation of tubulin abundance during vegetative growth and development (1, 7, 15, 25, 34, 45, 50). In multicellular organisms, several tubulin isogenes are usually expressed and at least some tubulin isotypes<sup>1</sup> appear to perform specialized functions. For example, in *Drosophila*, functions of at least two tubulin iso-

types cannot be substituted by transgenic expression of a different tubulin isotype (21, 33).

Because most cell types contain a mixture of different tubulin isotypes and multiple, distinct microtubule systems, tubulin isotype specialization could extend to the subcellular level. However, in most studies, cytoplasmic microtubules were found to contain a mixture of all tubulin isotypes expressed and thus no selective partitioning of endogenous tubulin isotypes was found (21, 29, 30, 33). Although a few cases of preferential partitioning of tubulin isotypes have been found either within the cell or in an in vitro polymerization assay (4, 9, 38), there is no direct evidence that these phenomena reflect intracellular partitioning of tubulin isotypes with specialized intracellular functions.

In most cell types, the primary tubulin gene products are further modified by secondary modifications (18, 36). Posttranslational tubulin modifications (PTM)<sup>2</sup> usually affect only a subset of the microtubules in a cell. It is intriguing that a small number of unicellular organisms which do not have detectable PTMs, such as fungi or *Dictyostelium discoideum*, also have relatively simple microtubular systems consisting largely of spindles and cytoplasmic microtubules (2, 50). This observation raises the possibility that,

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1. Dr. Gaertig's present address is Dept. of Cellular Biology, The University of Georgia, Athens, GA 30602-2607. Throughout this paper the term isoforms will be used to refer to different protein products of a single type ( $\alpha$  and  $\beta$ ) of tubulin that differ in electrophoretic mobility. The term isotypes will refer to the protein products of different ( $\alpha$  and  $\beta$ ) tubulin genes. Isoforms can be different secondarily modified products of a single ( $\alpha$  and  $\beta$ ) tubulin gene or can be derived from different genes.

2. Abbreviation used in this paper: PTM, posttranslational tubulin modification.

in organisms which express complex microtubular organelles, PTMs act in concert with mechanisms controlling tissue-specific isotype transcription to execute diverse functions of microtubules.

In general, a positive correlation exists between the stability of microtubule subpopulations and the extent of their secondary modification (16, 24, 28, 42, 51). However, despite intense study, the functional importance of even a single type of PTM remains unknown.

One of the best characterized PTMs is the acetylation of  $\alpha$ -tubulin at a conserved lysine at position 40 (10, 27). Although this modification is frequently associated with stable microtubules *in vivo*, it was found that isolated acetylated and unacetylated microtubules have similar stabilities *in vitro* (32). Thus, acetylation does not directly stabilize microtubules. To test the function of  $\alpha$ -tubulin acetylation *in vivo*, Kozminski et al. (26) expressed a mutant non-acetylatable  $\alpha$ -tubulin gene in *Chlamydomonas*. Up to 70% of endogenous tubulin was replaced without causing any detectable phenotypic alteration. However the 30% of wild-type acetylatable  $\alpha$ -tubulin still present in these transformed *Chlamydomonas* cells could have been sufficient to support the normal functions of acetylated microtubules.

In *Tetrahymena thermophila* there is only a single type of  $\alpha$ -tubulin gene (7, 35) and two genes that encode identical  $\beta$ -tubulin proteins (15). Thus, all of the (at least) 17 different microtubule systems in this ciliated protozoan are assembled using a single type of tubulin dimer primary sequence, providing one of the most striking examples of the multifunctionality of a single tubulin primary sequence. However, *Tetrahymena* tubulins are secondarily modified by several mechanisms, including acetylation, polyglutamylation (6, 40, 47) and possibly phosphorylation (20), to produce numerous tubulin isoforms (47). Thus, *Tetrahymena* provides an excellent system to study the function of PTMs in the absence of any complicating genetically encoded tubulin variation.

Recently, we described a method for homologous replacement of one of the two  $\beta$ -tubulin genes in *Tetrahymena* (14) in which a wildtype taxol resistant gene replaces a mutant taxol hypersensitive one. We have now developed a similar method for replacement of the single  $\alpha$ -tubulin gene in *Tetrahymena* (J. Gaertig, D. Pennock, and M. Gorovsky, unpublished results). Thus, the function of PTMs which occur either on  $\alpha$ - and  $\beta$ -tubulin can now be addressed directly in *Tetrahymena* by replacing the normal tubulin gene with a mutant version encoding a protein which can not be modified secondarily but which is otherwise wildtype. In this study, we begin our analysis of the functional significance of PTMs in *Tetrahymena* by completely eliminating acetylation of lysine 40 in  $\alpha$ -tubulin.

## Materials and Methods

### Cell Culture and Phenotypic Analyses of *Tetrahymena* Strains

*Tetrahymena thermophila* wild-type strain CU428, was obtained from P. Bruns (Cornell University, Ithaca, NY). *T. thermophila* strain E5 is an  $\alpha$ -tubulin mutant resistant to several tubulin-depolymerizing drugs (e.g., oryzalin and vinblastine) and hypersensitive to the microtubule-polymer-

izing drug taxol. The mutation responsible for this phenotype is an alanine to threonine replacement at position 65 in the  $\alpha$ -tubulin gene that will be described in detail elsewhere (J. Gaertig, D. Pennock, and M. Gorovsky, unpublished results). *Tetrahymena* were grown in SPP medium (17) with shaking at 30°C. Antibiotic-containing SPPA medium (12) was used for transformation, plating, and growth tests.

For growth experiments, 50 ml SPPA cultures in 250 ml Erlenmeyer flasks were inoculated to contain  $10^4$  cells/ml. Cells were grown at 30°C without shaking and periodically counted (Coulter Counter, model ZF; Coulter Electronics, Hialeah, FL). In some growth experiments cells were grown at different temperatures or with addition of NaCl or sucrose to the culture medium to test for phenotypic effects of tubulin acetylation. In the drug response experiments, cells were incubated in variable drug concentrations in 200  $\mu$ l of SPPA per well of a 96-well microtiter plate at 30°C, starting at  $10^4$  cell/ml density and counted after 24 h.

To measure the swimming rate, cells from growing cultures were observed using a 40 $\times$  lens under an Olympus BH2 microscope and photographed by exposing Kodak T-Max 400 film for 1 s. The negatives were projected using a photographic enlarger and paths of swimming cells were measured with a map reader. The absolute distances were calculated by calibrating with a stage micrometer.

To test pairing efficiency (39), the transformed clones were starved for 24 h in 10 mM Tris-HCl, pH 7.5. Cells (10 ml at a density  $2 \times 10^5$  cells per ml) of each tested strain were mixed with an equal number of untransformed wild-type (CU428) cells and incubated at 30°C. Samples of cells were withdrawn periodically, fixed with 1% (vol/vol) trichloroacetic acid and the fraction of total cells in pairs was determined.

### Immunodetection of Acetylated lys 40 Tubulin Epitope

Monoclonal antibody 6-11B-1, which is specific for a conserved epitope surrounding the acetylated lysine at residue 40 on  $\alpha$ -tubulin in *Chlamydomonas* (41), was used to detect acetylated  $\alpha$ -tubulin. This antibody was generously provided by Dr. G. Piperno (Rockefeller University, New York, NY). As a general anti- $\alpha$ -tubulin probe we used the DM1A monoclonal antibody (Amersham Corp., Arlington Heights, IL). Immunofluorescent detection of tubulins was performed as described previously (11) except that 1  $\mu$ M taxol was added to the cytoskeleton extraction buffer to help retain the cytoskeleton in the extracted cells. The 6-11B-1 and DM1A antibodies were used at 1/5 and 1/500 dilution, respectively.

### Plasmid Vectors

The pTUB100 plasmid contains the 3.2-kb HindIII genomic fragment of the  $\alpha$ -tubulin gene of *T. thermophila* (35). pTUB100E3 (see Fig. 2) is a derivative of pTUB100 in which a HaeIII restriction site has been introduced by a silent substitution at alanine codon 281 (GCT to GCC). The pTUB-R40 is a derivative of pTUB100E3 where an arginine codon at position 40 was introduced in place of the wild-type lysine codon (AAG codon to AGA). All plasmid alterations were done using site-directed mutagenesis according to (54).

### Transformation by Gene Replacement

For gene replacement, plasmid DNA was extracted as previously described (12). Either pTUB100E3 or pTUB-R40 plasmid DNA was digested with HindIII restriction endonuclease to separate insert from vector and to create homologous insert ends. The digested DNA was purified by phenol/chloroform/isoamyl alcohol (25:24:1) extraction followed by chloroform/isoamyl alcohol extraction (24:1), precipitated with ethanol, dried and resuspended in TE buffer at 250  $\mu$ g/ml. Vegetative E5 cells were transformed by microinjection of plasmid DNA into the macronucleus (48). After microinjection, cells were cloned into small drops of SPPA medium, grown to saturation, and transferred on 15  $\mu$ M taxol in SPPA. Transformed clones resistant to taxol were isolated after 2–3 d.

### Analysis of DNA

Total DNA was isolated from growing *Tetrahymena* cultures and Southern blots were prepared as described previously (14). The  $\alpha$ -tubulin-specific probes (see Fig. 2) were either the 1-kb BglII fragment of the coding sequence (probe A) or the entire cloned 3.2-kb HindIII fragment of the *T. thermophila*  $\alpha$ -tubulin gene (probe B) (35). Probes were prepared by random-primer extension using [<sup>32</sup>P]dATP labeling (3). Hybridization was performed under stringent conditions as described (31).

## Protein Electrophoresis and Immunoblotting

To extract total proteins,  $3 \times 10^5$  cells *Tetrahymena* from a mid logarithmic phase culture were pelleted by centrifugation at 1600 g for 1 min, resuspended in 1 ml of 10 mM Tris-HCl, pH 7.5, and further concentrated to a dense pellet by a 30-s spin in a microfuge. After removing the supernatant, the cell pellet was quickly resuspended in 300  $\mu$ l of boiling 2 $\times$  SDS sample buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, 0.001% Bromophenol blue, 10%  $\beta$ -mercaptoethanol) and boiled for 5 min. Protein extracts were run on 10% SDS-polyacrylamide gels. Duplicate gels were blotted (19, 49) to Immobilon-P PVDF membranes (Millipore Corp., Bedford, MA) using a Trans-Blot semi-dry transfer cell (Bio-Rad Labs., Richmond, CA) according to the manufacturer's instructions, and a modified transfer buffer (48 mM Tris base, 39 mM glycine, 0.037% SDS and 15% methanol). Blots were blocked in 1% BSA in TBS buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.1). For antibody incubation, TBS with 1% BSA and 0.05% Tween 20 was used. Incubations using the primary antibodies were done at 4°C overnight. The anti- $\alpha$ -tubulin antibody (Amersham) and the 6-11B-1 anti-acetylated tubulin antibody were used at 1/1,000 and 1/67 dilution, respectively. As a secondary antibody, peroxidase conjugated rabbit anti-mouse antiserum (1:1,500 dilution, Zymed Labs. Inc., South San Francisco) was used. Blots were developed with HRP Color Development Reagent (Bio-Rad) according to manufacturer's instructions.

## Isoelectric Focusing Analysis of Axonemes

Cilia were isolated and the axonemal proteins were extracted (22) and analyzed on isoelectric focusing gels prepared according to Bolduc et al. (5).

## Results

### Distribution of Microtubules Containing Acetylated $\alpha$ -Tubulin in Vegetative *Tetrahymena*

The distribution of  $\alpha$ -tubulin acetylated at lysine 40 (lys 40) in wild-type vegetative cells was analyzed using the monoclonal antibody 6-11B-1. By immunofluorescent analysis, we found the 6-11B-1 epitope in most, but not all, of the previously characterized microtubule systems in *Tetrahymena*. The antibody reacted strongly with most, if not all, microtubule structures present in the cell cortex, including the transverse fibers, postciliary microtubules, and longitudinal microtubular bundles (Fig. 1 A). Because of the proximity of stained microtubule band structures (transverse and postciliary), we could not determine if basal bodies were stained by 6-11B-1. On the posterior/dorsal side of the cell cortex, the rosette-like openings of the contractile vacuole pore complexes were stained (Fig. 1 A). On the ventral surface, the oral apparatus and the associated postoral microtubular bundle were detected (Fig. 1 C). Under standard fixation conditions only tips of the cilia were stained (Fig. 1, A and C). However, the entire lengths of cilia were strongly reactive to 6-11B-1 when cells were dried on a slide before antibody staining (Fig. 1 B), as was found for *Chlamydomonas* flagella (26). In dividing cells, the primordium of the new oral apparatus forms in the equatorial region and it was also detected by 6-11B-1 (Fig. 1 C). The acetylated epitope also was found associated with intramicronuclear division spindles of dividing cells (Fig. 1, C and D). We did not detect any macronuclear staining. However, intramacronuclear microtubules could be visualized using an anti-sequence  $\alpha$ -tubulin antibody DM1A (data not shown), suggesting that acetylated tubulin is absent from the somatic macronucleus as opposed to the micronucleus. The 6-11B-1 antibody did

not react with the cytoplasmic network of microtubules, which can be visualized by other types of antitubulin antibodies in vegetative and conjugating cells (11).

### Transformation of *Tetrahymena* with a Mutant Gene Encoding Nonacetylatable $\alpha$ -Tubulin

To test the function of  $\alpha$ -tubulin acetylation at lys 40, we replaced the single  $\alpha$ -tubulin gene with a mutant gene encoding nonacetylatable tubulin. The E5 mutant strain of *T. thermophila* was used as the host cell for  $\alpha$ -tubulin gene replacement. E5 is a nitrosoguanidine-induced germ-line mutant of *Tetrahymena* isolated by selection for oryzalin resistance and taxol sensitivity (J. Gaertig, D. Pennock, and M. Gorovsky, manuscript in preparation). In the E5  $\alpha$ -tubulin gene, codon 65 encodes threonine instead of the normal alanine. Because the E5 strain is also hypersensitive to taxol, it can be transformed by gene replacement to taxol resistance using a wildtype tubulin gene. We used the cloned 3.2 kb HindIII genomic fragment containing the *Tetrahymena*  $\alpha$ -tubulin gene to prepare two types of transforming fragments (Fig. 2 A). The plasmid pTUB-R40 contains the  $\alpha$ -tubulin gene mutagenized so that codon 40 encodes arginine (arg) instead of lysine (lys). Thus, pTUB-R40 encodes a nonacetylatable  $\alpha$ -tubulin. We also introduced a silent HaeIII restriction site into pTUB-R40 to facilitate analysis of DNA in transformed cells. As a control containing an acetylatable tubulin gene, we constructed pTUB100E3 containing only the HaeIII site. Both constructs contain an alanine codon at position 65 and thus can transform the E5 strain (containing threonine 65) to taxol resistance/oryzalin sensitivity by gene replacement in the macronucleus (Fig. 2 A). Usually gene replacement occurs only in the somatic macronucleus. However, since micronuclear genes are not expressed in vegetative cells, they do not contribute to the phenotype.

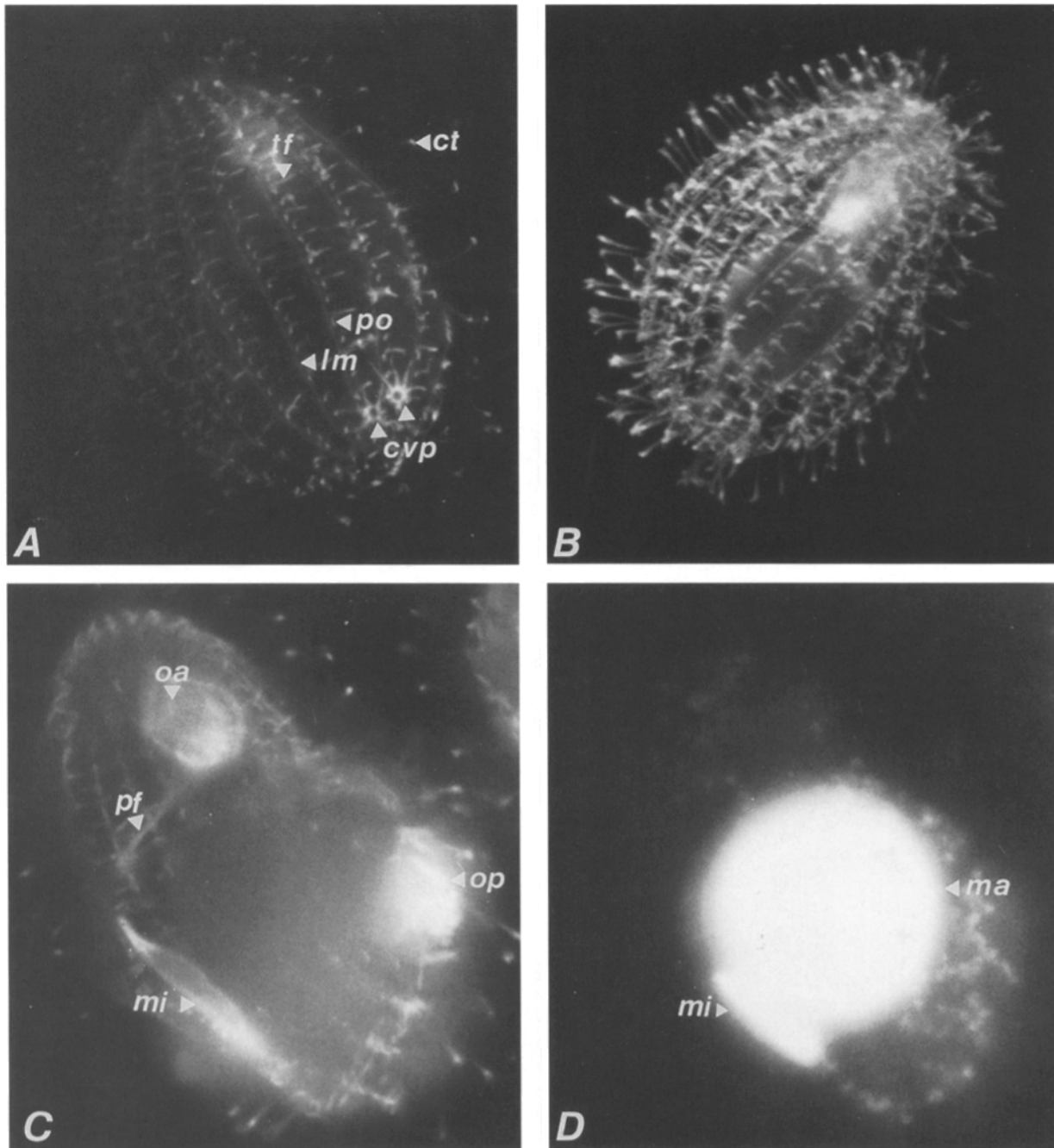
Because the E5 strain is sterile and cannot be transformed by electroporation of conjugants (12), each construct was introduced into the macronucleus of strain E5 by microinjection of  $\sim$ 200 cells. Injected cells were grown for  $\sim$ 20 generations and then selected for taxol resistance. Seven and five putative transformants, respectively, were obtained using pTUB-R40 and pTUB100E3. No taxol-resistant cells were obtained in 96 clones derived from noninjected cells. All putative transformants grew at 15–25  $\mu$ M taxol, which completely inhibits growth of the E5 host. All transformants, regardless of the type of transforming gene (lys or arg 40) grew at similar rates on the SPPA-selective medium containing taxol. All transformants were also oryzalin and vinblastine sensitive (failed to grow at 30  $\mu$ M concentration of either of the two drugs) consistent with transformation by a wild-type (ala 65)  $\alpha$ -tubulin gene.

### Taxol-resistant Transformants Obtained Using Either arg or lys 40 Fragments Underwent Gene Replacement within the $\alpha$ -Tubulin Locus

To reveal the mechanism of transformation of the E5 strain to taxol resistance/oryzalin sensitivity, we extracted genomic DNA from seven arg 40 transformants (R1-R7 strains) and from one control lys 40 transformant (K1 strain) 20 generations after transformation and analyzed it by Southern blotting (Fig. 2 B). In the wildtype strain,

HaeIII digestion yields a single 8.8-kb fragment detected by the  $\alpha$ -tubulin probe. Due to the additional HaeIII site in the transforming DNA, HaeIII digestion of transformants will produce two fragments of 5.5 and 3.3 kb if gene

replacement occurred within the  $\alpha$ -tubulin locus (Fig. 2 A). As shown in Fig. 2 B, in the host strain E5, HaeIII digestion produced one major band of  $\sim 9$  kb, as expected. In all transformants, two new fragments of  $\sim 5.5$  and  $\sim 3.3$

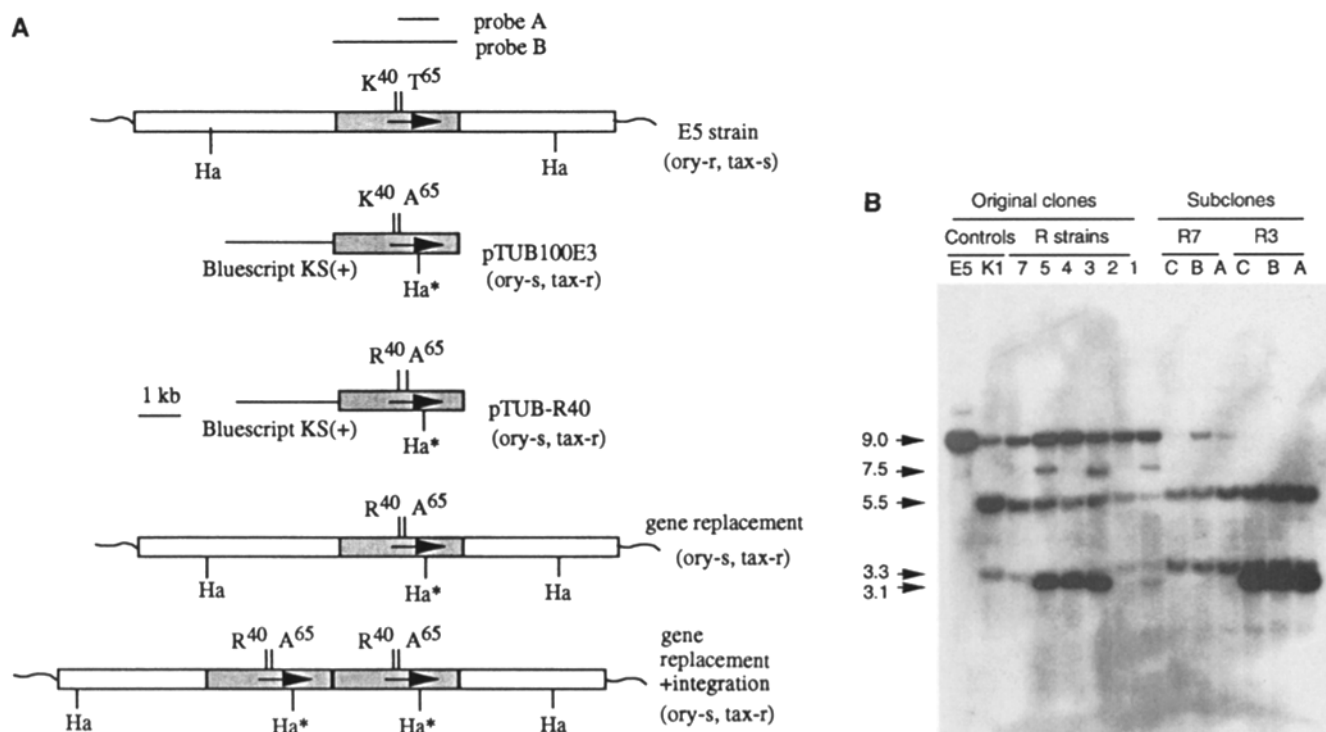


**Figure 1.** Distribution of acetylated  $\alpha$ -tubulin in *Tetrahymena* detected using the 6-11B-1 monoclonal antibody. Growing cells were analyzed using immunofluorescence. (A) Dorsal surface of the cell. All classes of microtubule-containing cortical organelles including longitudinal microtubule bundles, transverse fibers and postciliary microtubules are stained. In the posterior part of the cell the two rosette-like structures are the contractile vacuole pore complexes which stain heavily. Note that only the tips of the cilia are stained using standard immunofluorescence. (B) A cell that was dried on a coverslip before immunostaining. In addition to the cortical microtubule system, cilia are intensely stained along their entire length. (C) A cell undergoing division, stained using standard conditions (without drying). In addition to the old oral apparatus, a new oral apparatus primordium is forming in the equatorial region of the cell and it contains the acetylated tubulin epitope. The postoral microtubule system also stains. The acetylated epitope is also associated with the micronuclear mitotic spindle. (D) distribution of DNA (stained by DAPI) in the cell shown in C. *tf*, transverse fibers; *po*, postciliary microtubules; *lm*, longitudinal microtubule bundle; *cvp*, contractile vacuole pore; *ct*, ciliary tips; *pf*, postoral microtubule fiber; *oa*, oral apparatus; *op*, oral apparatus primordium; *mi*, micronucleus; *ma*, macronucleus.

kb were present, consistent with  $\alpha$ -tubulin gene replacement (Fig. 2 B, original clones). However, the 8.8-kb band corresponding to the endogenous gene was still present in all transformants, demonstrating that only partial replacement of genes in the polycopy macronucleus occurred initially. In addition to the two fragments diagnostic for gene replacement, four of the transformants contained an additional HaeIII fragment of  $\sim 3.1$  kb (Fig. 2 B, clones R1, 4, 5, 7) that represents an additional copy of the transformed gene inserted into the flanking region of the  $\alpha$ -tubulin gene. This type of homologous recombination event was previously observed for gene disruption transformants at the HHF1 locus encoding histone H4 (13, 23) and the HTA3 locus encoding a histone H2A variant (X. Liu and M. Gorovsky, unpublished data).

### The Endogenous Gene Encoding Acetyltable $\alpha$ -Tubulin Is Eliminated in Subclones of *arg 40* Transformants

Because the *Tetrahymena* macronucleus contains  $\sim 45$  copies of each gene, only a partial replacement of some of the endogenous gene copies initially occurs in a typical gene replacement experiment (13, 14, 23, 53). In our transformants, the endogenous  $\alpha$ -tubulin gene fragment was still present in transformants  $\sim 20$  generations after transformation (Fig. 2 B, original clones). However, during vegetative growth, the macronuclear allelic copies are randomly sorted between two daughter cells, a phenomenon known as phenotypic assortment (37). If the endogenous gene is not essential, then during additional growth under negative selective pressure it can be completely replaced



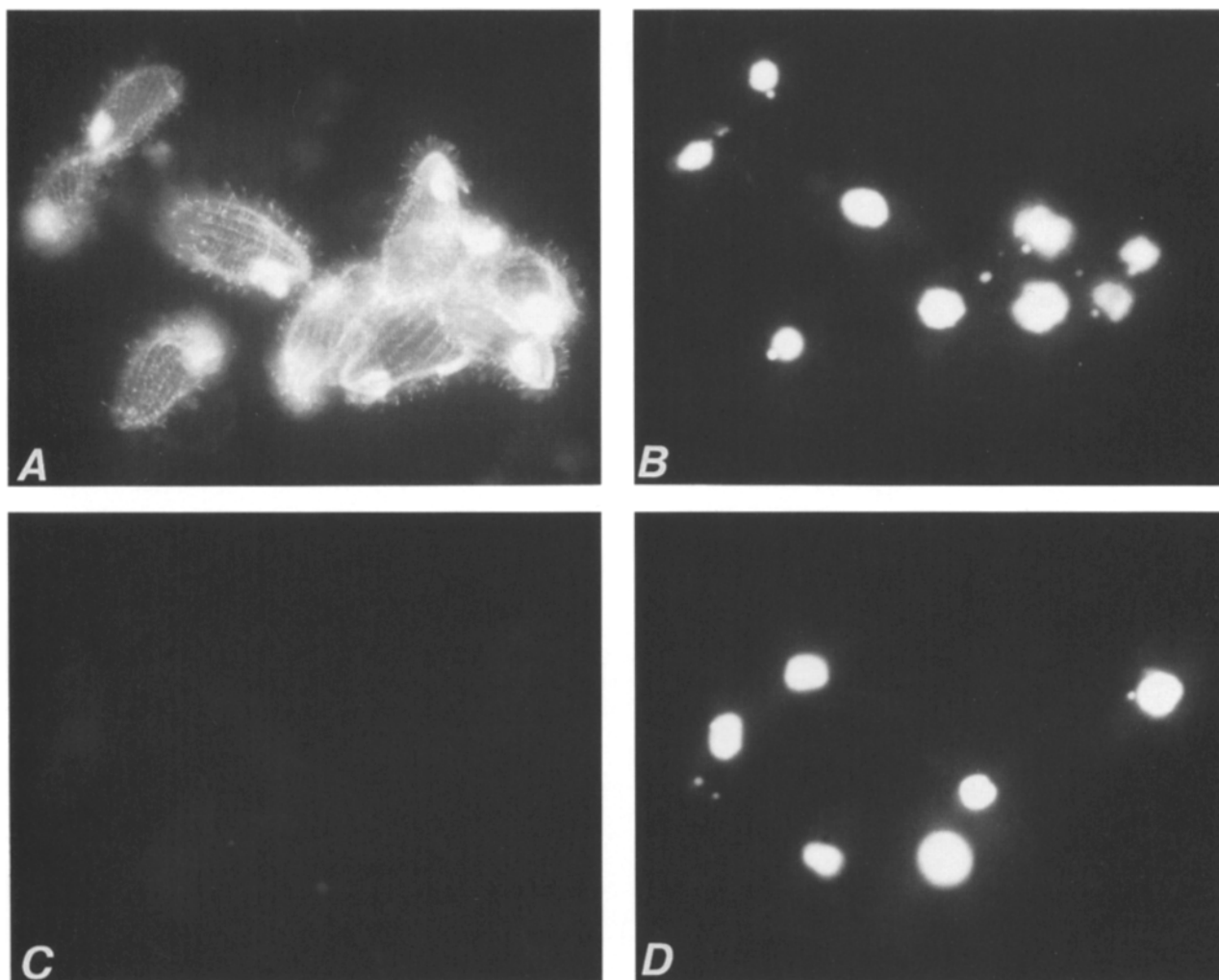
**Figure 2.** (A) Diagrammatic representation of the genomic structure of the  $\alpha$ -tubulin locus and the experimental design of gene replacement. The open bar is the genomic region flanking the  $\alpha$ -tubulin gene. The shadowed bar is the cloned 3.2-kb HindIII fragment of the  $\alpha$ -tubulin gene. The arrow shows the position and transcriptional orientation of the coding sequence of the  $\alpha$ -tubulin gene. The thin line represents vector sequence. Positions of the DNA probes are marked at the top of the figure.  $K^{40}$ , lysine codon at position 40;  $T$ , threonine codon;  $A$ , alanine codon;  $R$ , arginine codon;  $Ha$ , HaeIII restriction site;  $Ha^*$ , vector specific HaeIII restriction site. (B) Southern blot analysis of genomic DNA isolated from transformants and control strains. Total DNA was isolated, digested with HaeIII restriction endonuclease, separated on a TAE-agarose gel, blotted, and probed with radiolabeled probe B (see Fig. 2 A). The original transformant clones were analyzed 20 generations following microinjection. Three subclones (designated A, B, and C) were derived from the R7 and R3 transformant strains after additional growth for 50–60 generations. The endogenous (host-specific) genomic HaeIII fragment has a size of  $\sim 8.8$  kb. When this gene is replaced, incorporation of the vector-specific HaeIII site should yield two new fragments of 3.3 and 5.5 kb. These two fragments diagnostic for gene replacements are detected in all transformants. In addition, in four out of six R strains, an additional fragment of  $\sim 3.0$  kb in size is observed. Based on additional restriction mapping and PCR analysis of the transformants DNA (results not shown) it represents an additional copy of the transformed gene integrated into the flanking region of the  $\alpha$ -tubulin gene. The 7.5-kb band observed in some lanes probably represents a partially digested fragment. Amounts of the endogenous gene (9.0 kb) are greatly reduced or undetectable in subclones made from original transformants, thus the endogenous gene is assorted in the transformants subjected to additional growth. The remaining small amount of 8.8-kb DNA corresponding to the endogenous gene present in some subclones could also be partially digested transformed gene. The micronuclear fragment containing the  $\alpha$ -tubulin gene is larger than the macronuclear copy due to the presence of flanking micronucleus-specific sequences (7). Because the micronuclear DNA represents only  $\sim 5\%$  of the total *Tetrahymena* DNA, it is not readily detectable on this Southern blot.

by assortment (14). We attempted to complete gene replacement in arg 40 transformants R3 and R7 by growing cells in 15  $\mu$ M taxol-containing medium for 50–60 generations. R7 is a gene replacement transformant while R3 contained an additional integrated copy of the arg 40 gene (see above). The K1 transformant containing lys 40 was included as a control. All strains tested grew at similar rates during the entire additional selection period. At the end of selection, total DNA was extracted from all strains, digested with HaeIII and analyzed on a Southern blot. This time, no endogenous gene was found in some transformants, consistent with complete replacement of all  $\sim$ 45 endogenous copies of the  $\alpha$ -tubulin gene (Fig. 2 *B*, *subclones*). Thus the acetylatable  $\alpha$ -tubulin gene is not essential in *Tetrahymena* and it can be replaced by a nonacetylatable gene containing arg instead of lys 40.

**Arg 40 Complete Replacement Transformants Produce No Detectable Acetylated  $\alpha$ -Tubulin Protein**

We used the 6-11B-1 antibody to analyze the acetylatable tubulin in transformants using immunofluorescence. In a

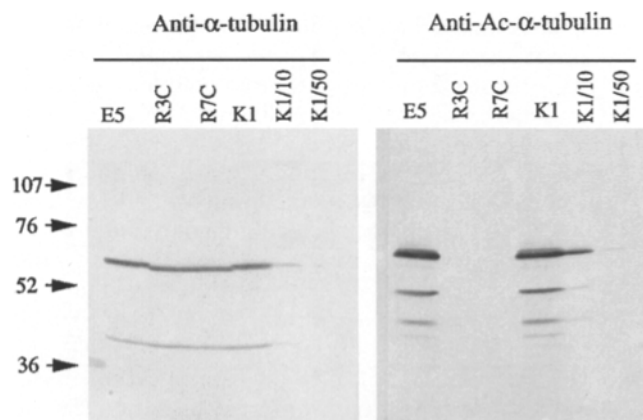
lys 40 control transformant (K1), the 6-11B-1 antibody produced strong staining of microtubular structures (Fig. 3, *A* and *B*) as expected. In contrast, both arg 40 clones selected by phenotypic assortment (R7A and R3A) were negative for 6-11B-1 antibody staining (Fig. 3, *C* and *D*, and data not shown for R3A). To quantitate the amounts of acetylated tubulin, we analyzed total proteins from transformants probed with 6-11B-1 antibody on a Western blot. Normal levels of 6-11B-1 reactive  $\alpha$ -tubulin were found in the K1 (lys40) transformant while no detectable staining was found in two arg 40 (R3C and R7C) transformants. We could detect a signal of the acetylated tubulin using only a 1/50 dilution of the control strain extract (Fig. 4). Thus, we should be able to detect 2% or more of the normal content of acetylated tubulin in transformants. Because there are  $\sim$ 45 copies of each gene in the somatic macronucleus (52), R3C and R7C strains contain less acetylated tubulin than would be expected from one gene copy per macronucleus; thus they are complete gene replacement transformants. In contrast to the 6-11B-1 antibody, the DM1A anti- $\alpha$ -tubulin antibody detected similar levels of  $\alpha$ -tubulin in both arginine and lys 40 transform-



**Figure 3.** Immunofluorescent analysis of transformants. Growing cells were immunostained with the 6-11B-1 primary antibody and anti-mouse IgG-FITC conjugate as a secondary antibody (*A* and *C*) and counterstained with DAPI (*B* and *D*) to visualize the DNA in each cell. (*A* and *B*) Cells from the control transformed strain K1, containing the wild-type lys 40 codon in the  $\alpha$ -tubulin gene. (*C* and *D*) Cells of the R7C transformant containing the arg 40 codon.

ants (Fig. 4). Thus, arg 40 transformants produce normal amounts of  $\alpha$ -tubulin which remains unacetylated. Interestingly, the nonacetylated  $\alpha$ -tubulin present in the arg 40 strain samples migrates slightly faster than most of the  $\alpha$ -tubulin in the control strains. Acetylation apparently reduces mobility of  $\alpha$ -tubulin on SDS-PAGE gel. It is also clear that nearly all  $\alpha$ -tubulin in the control lys 40 strains migrates more slowly suggesting that most of the  $\alpha$ -tubulin is acetylated in *Tetrahymena*. These biochemical data are consistent with our immunofluorescent results (see above) that indicate most microtubule systems in *Tetrahymena* contain acetylated tubulin.

We also analyzed tubulins using isoelectric focusing. To obtain material highly enriched in tubulin, we isolated cilia from vegetatively growing cells and extracted axonemal proteins. About five to seven differently charged  $\alpha$ -tubulin and two or three  $\beta$ -tubulin isoforms can be separated (Fig. 5) as previously described (47). In the arg 40 transformants a large shift in positions of most bands in the  $\alpha$ -tubulin region toward the basic end of the gel occurred, consistent with the absence of lysine acetylation in those transformants. This shift in mobility of the entire  $\alpha$ -tubulin staining pattern indicates that most if not all ciliary  $\alpha$ -tubulin is acetylated in the normal strain. Furthermore, the fact that several distinct  $\alpha$ -tubulin bands are still present in the acetylation-minus strain argues that one or more types of other secondary modifications that alter the charge of  $\alpha$ -tubulin still occur in those strains and must coexist with acetylation on the same tubulin molecules in normal



**Figure 4.** Western blot analysis of E5 and transformed clones. Equal amounts of total protein extracts from growing cells as well as 1/10 and 1/50 dilution of the control transformant extract K1 were separated on 10% PAGE and duplicate immunoblots were probed using either the DM1A anti- $\alpha$ -tubulin antibody (left panel) or the 6-11B-1 antibody against the acetylated  $\alpha$ -tubulin. The general antibody DM1A detects similar amounts of  $\alpha$ -tubulin in all extracts from all transformant strains. In contrast, the 6-11B-1 antibody detects acetylated tubulin only in the control transformant K1 and in the host strain E5. The acetylated epitope is detected in the K1 control transformants even if only 1/10 or 1/50 of the protein extract was loaded (last two lanes to the right). Thus, the R3C and R7C strains analyzed here contain less than 1/50 of the normal level of acetylated  $\alpha$ -tubulin. Because there are  $\sim 45$  copies of each gene in the polyploid macronucleus, R3C and R7C are complete transformants. The bands running faster than tubulin are probably proteolytic fragments of tubulin.

strains. This result also demonstrates that, in the arg 40 transformant, no acetylation at one of the remaining lysine residues (or no other modification) occurred to compensate for the absence of lys 40 acetylation. We did not observe any incorporation of radioactive acetate into  $\alpha$ -tubulin in the arg 40 strain (in the presence of cycloheximide);  $\alpha$ -tubulin was radiolabeled in the lys 40 strain under these conditions (results not shown).

#### ***Tubulin Acetylation-deficient Cells Have Normal Morphology and Assemble the Same Microtubule-containing Structures as Wild-type Cells***

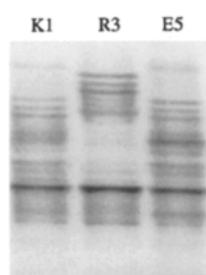
Arg 40 and lys 40 transformants are similar in general appearance. They have similar sizes, number of cilia, and ciliary rows as determined by SEM microscopy (results not shown). Staining with a general antitubulin monoclonal antibody revealed that arg 40 strains maintained normal microtubule containing structures: ciliary axonemes, cortical fibers, and oral apparatus. Normal staining of basal bodies, oral apparatus, and postoral structures was observed with the monoclonal antibody MPM-2 (data not shown), which reacts with microtubule organizing centers containing one or more phosphorylated epitopes in many cell types (8) including *Tetrahymena* (46 and our unpublished observations). Thus, strains deficient in acetylated tubulin have morphologically normal microtubule-containing structures.

#### ***Strains Containing No Acetylated $\alpha$ -Tubulin Display Wild-type Growth Characteristics***

No differences in growth rates or final culture densities between arg 40 and lys 40 strains were observed in cells grown at 18, 30, or 39°C, in media whose osmotic pressure was modified by addition of 0.1 or 0.2 M sucrose, or in media containing 0.5% or 0.75% NaCl (data not shown).

#### ***Cilia-dependent Motile Functions in Strains Lacking Acetylated Tubulin Are Indistinguishable from Wild-type Strains***

Since cilia contain high concentrations of acetylated tubulin, we examined a number of functions in which cilia are involved. No significant difference in swimming velocity



**Figure 5.** Analysis of axonemal proteins using an isoelectric focusing gel. Growing cells were deciliated, cilia were isolated and axonemal proteins extracted and run on an isoelectric focusing gel. At least five isoforms of  $\alpha$ - and two to three isoforms of  $\beta$ -tubulin can be distinguished in the tubulin region of the gel (marked on the right side of the figure). The mobility of all tubulins is similar in the K1 control trans-

formant and in the E5 host strain. However in the R3 (arg 40) strain, the entire  $\alpha$ -tubulin region composed of about five bands is shifted toward the more basic part of the gel (top). This result is consistent with a decrease in acidity of nonacetylated tubulin in the R3 strain.

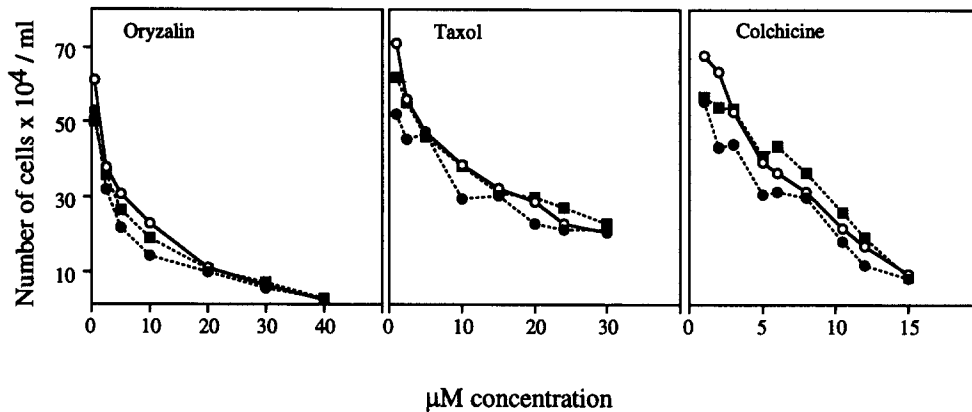


Figure 6. Effect of antitubulin drugs on survival of transformed strains. Equal numbers of cells of each tested strain were mixed with an equal volume of SPPA medium containing variable amounts of antitubulin drugs: oryzalin, colchicine, or taxol. Cells were incubated for 24 h at 30°C in microtiter plate wells and counted using a Coulter Counter. Data are from a single experiment. No differences between the strains were found in an additional independent experiment. ■, R3C strain; ●, R7C strain; ○, K1 strain.

among tested strains was found. Lys 40 cells (K1) swam at  $290 \pm 142 \mu\text{m/s}$  ( $n = 52$ ) while arg 40 cells (R3C and R7C) swam at  $286 \pm 174 \mu\text{m/s}$  ( $n = 69$ ). No obvious difference in the feeding rate between arg 40 and lys 40 strains was found and no differences in pair formation kinetics between tested arg and lys 40 strains were observed (data not shown). Thus, functions dependent on cilia are not detectably affected by elimination of acetylated tubulin in *Tetrahymena*.

#### Cells Lacking Acetylated Microtubules Display Wild-type Sensitivities to Antitubulin Drugs

If acetylation of tubulin increases microtubule stability, then cells unable to undergo acetylation might have more labile microtubules and be more sensitive to microtubule-depolymerizing drugs such as oryzalin, or more resistant to the microtubule-stabilizing drug taxol. We incubated cells with different concentrations of antitubulin drugs and counted cells after 24 h. No differences in drug sensitivities were found between arg and lys 40 strains (Fig. 6). In the presence of antitubulin drugs (such as oryzalin) *Tetrahymena* gradually resorb cilia and lose motility. If ciliary axonemes containing acetylated tubulin are more stable, then the rate of their resorption should be slower in lys 40 strains than in arg 40 strains lacking acetylated tubulin. No differences were found between the transformed strains when we incubated starved cells in the presence of oryzalin and periodically inspected the number and length of cilia using SEM (data not shown).

#### Discussion

Ciliates represent a case of remarkable microtubule diversity. In a single cell they maintain as many different microtubular structures as multicellular organisms do in an entire organism. In ciliates, tubulins also are as extensively modified as in multicellular organisms. However, ciliates have little or no tubulin primary sequence variability, and from this point of view are more similar to some fungi, flagellates and the slime mold *Dictyostelium*. Somatic transformation was used to replace all  $\sim 45$  copies of the

single  $\alpha$ -tubulin gene in the *Tetrahymena* macronucleus with a gene encoding a nonacetylatable tubulin, creating viable *Tetrahymena* cell lines that do not express any detectable  $\alpha$ -tubulin acetylated at lys 40. The absence of acetylated tubulin was demonstrated biochemically and by immunocytochemistry with a monoclonal antibody highly specific for the acetylated lys 40 epitope. The absence of lys 40 acetylation not only has no effect on microtubule stability in vivo (measured as a level of resistance to tubulin depolymerizing drugs) but also has no detectable effect on many aspects of cell function that involve microtubules, including growth under various conditions, swimming, feeding and mating. The fact that  $\alpha$ -tubulin acetylation at lys 40 is not essential in *Tetrahymena thermophila*, with its highly diverse microtubules and large amounts of acetylated  $\alpha$ -tubulin, suggests it is not likely to be essential in other organisms as well.

Only a few organisms (including yeast and *Dictyostelium*) that contain relatively simple microtubule systems have no lysine at position 40 in  $\alpha$ -tubulin and therefore do not contain an acetylated tubulin isoform (2, 50). To our knowledge, there are only two cases of cell types that contain an  $\alpha$ -tubulin with lysine at position 40 but lack detectable acetylation at that site: turkey erythrocytes (44) and *Plasmodium falciparum* (43). In the case of turkey erythrocytes, the microtubule bands present in these cells are highly stable, demonstrating that microtubule stabilization can be achieved without tubulin acetylation in vivo. Thus, although lys 40 acetylation is widespread and evolutionarily conserved, a few organisms and cell types manage to survive without it.

If lys 40 acetylation is not essential then why it is evolutionarily conserved? There are several possible explanations for this surprising result. It is possible that our laboratory tests failed to detect a subtle function of this modification. A very slight increase in fitness of an organism containing modified tubulin may provide a significant advantage in nature or on an evolutionary time scale but be undetectable in the laboratory. It is also possible that  $\alpha$ -tubulin acetylation has a function that is redundant with that of one or more other PTMs. In most cells, tubulins are modified by several different mechanisms. This is clearly



the case in *Tetrahymena* axonemes where virtually all molecules that are acetylated contain at least one other secondary modification as well (Fig. 5). Thus, in the absence of one type of PTM such as  $\alpha$ -tubulin acetylation, the remaining modification(s) may still provide necessary functions. The hypothesis that different PTMs can have redundant function can be tested in *Tetrahymena* by transforming with tubulin genes in which the sites of different PTMs have been simultaneously inactivated.

Absence of essential PTM function may be compensated by qualitative or quantitative changes in unknown factors such as microtubule associated proteins (MAPs) that interact with PTMs in vivo. If the function of a PTM is related to microtubule disassembly or to tubulin protein degradation, absence of the modification could lead to increased half-life of cytoplasmic microtubules and may be compensated by a reduced rate of depolymerization, by a reduction in tubulin production, or by increased activity of a protein degradation pathway. Examples of such compensatory changes have been observed in strains of *Tetrahymena* where single genes were inactivated by a gene disruption. For example if either of two  $\beta$ -tubulin genes are eliminated, the level of mRNA from the remaining gene increases to restore the normal level of  $\beta$ -tubulin mRNA (L. Gu, unpublished results). Further biochemical studies may reveal any changes in the amounts of MAPs or changes in turnover rates of tubulin proteins or mRNA in the acetylation deficient strains.

It should also be considered that acetylation of lys 40 in  $\alpha$ -tubulin is without function. If this is the case, its evolutionary conservation needs explanation. It is possible that lys 40 plays an important role in tubulin function for reasons unrelated to its acetylation. The requirement of lysine at this position can now be tested in *Tetrahymena* by replacing this residue with another one not as structurally similar to lysine as arginine. Partial replacement of lysine with alanine at this position in up to 70% of total tubulin in *Chlamydomonas* (26) was not sufficient to cause phenotypic changes. This experiment can be repeated in *Tetrahymena* using a complete gene replacement. It also should be noted however that, if lysine is conserved at this position for reasons unrelated to acetylation, and arginine functions at position 40 as well as lysine (as indicated by our results), it is surprising that arginine is not found frequently at this position in naturally occurring  $\alpha$ -tubulin.

If lys 40 in  $\alpha$ -tubulin is conserved for reasons unrelated to its modification, and its acetylation is not essential, then one would expect to find organisms that contain lys 40 and are no longer able to perform acetylation. Although, as noted above, acetylation is not detected at least in two cell types that express lys 40  $\alpha$ -tubulin, in most cells tubulins having lys 40 are acetylated at this position judging by their reactivity to acetylation-specific antibodies. This suggests that tubulin acetyltransferase occurs in those cells. If lys 40 is conserved for a reason other than acetylation and tubulin acetylation itself is not essential, tubulin acetyltransferase activity could be essential for reasons unrelated to tubulin modification. For example, tubulin acetyltransferase may have another target, structurally similar to the  $\alpha$ -tubulin lys 40 acetylation recognition site, whose acetylation may be essential or strongly advantageous. The possibility that tubulin acetyltransferase activity is es-

sential in modification of an unknown protein can be investigated by cloning the *Tetrahymena* tubulin acetyltransferase gene and knocking it out using newly developed techniques for gene disruption. If the sole function of tubulin acetyltransferase is to acetylate tubulin, knocking it out should mimic the effect of the arginine replacement of lys 40 and give no detectable phenotype using the assays described here. If the acetyltransferase performs a function in addition to acetylating  $\alpha$ -tubulin, knocking it out could be lethal or could give a distinct phenotype.

In summary, we have demonstrated that the complete absence of a conserved secondary modification, acetylation of lysine at residue 40 in  $\alpha$ -tubulin, is without detectable phenotype in *Tetrahymena*. We have also established a general approach whereby the endogenous tubulin genes in this organism can be completely replaced with otherwise wildtype genes lacking specific sites of secondary modification. Hopefully, this approach can be used in the future to study the function(s) of all of the secondary modifications of tubulin both individually and in combination.

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