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Genetics

Electroporation-mediated replacement of a positively and negatively selectable β-tubulin gene in *Tetrahymena thermophila*

(gene replacement/microtubule drugs/colchicine/oryzalin/taxol)

JACEK GAERTIG, THOMAS H. THATCHER, LONG GU, AND MARTIN A. GOROVSKY

Department of Biology, University of Rochester, Rochester, NY 14627

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ABSTRACT **Replacement of lysine-350 by methionine in the β-tubulin gene of *Chlamydomonas* confers resistance to microtubule-depolymerizing drugs and increased sensitivity to the microtubule-stabilizing drug taxol. This mutation was created in cloned BTU1, one of two coexpressed β-tubulin genes of *Tetrahymena thermophila*. When introduced by electroporation, the mutated gene transformed *Tetrahymena* exclusively by gene replacement at the homologous locus. Taxol-sensitive transformants could be retransformed with a wild-type gene and selection for taxol resistance. Analyses of phenotypic assortment and of the mRNA in transformed cells suggest that complete replacement of the BTU1 gene in the polyploid macronucleus can be obtained. These studies demonstrate the utility of this marker for studying tubulin gene function and show that electroporation allows facile gene replacement in *Tetrahymena*.**

Microtubules are polymers of a heterodimer of highly conserved α- and β-tubulin. They are involved in cell and organelle motility, provide the structural framework for membranous and other cytoskeletal elements, and are involved in localizing cytoplasmic determinants during development. While some organisms have multiple α- and/or β-tubulin isotypes that may have cell type-specific function, in others, diverse microtubule structures are constructed from a single α and a single β isotype (1). Conserved modifications (acetylation, phosphorylation, tyrosination, glutamylation) of tubulin may contribute to the structural and functional diversity of microtubules (see ref. 2 for review). However, the function of even a single modification has yet to be established.

One approach to analyzing the role of tubulin secondary modification is to knock out or mutate the modifying enzymes or their target sites on the tubulins. However, with one recent exception (3), genes encoding the modifying enzymes have not been cloned. In higher eukaryotes, which usually contain multiple tubulin genes, it is difficult to replace all of the expressed tubulin genes with a mutated gene. Also, in multicellular organisms targeting of transforming DNA to the correct locus is difficult. Alternatively, in some lower eukaryotes, particularly in the fungi, gene replacement is readily accomplished but microtubule systems are relatively undifferentiated and many of the secondary modifications found in multicellular organisms do not occur.

We are developing the ciliate *Tetrahymena thermophila* as a model for studying microtubule function. Although unicellular, *Tetrahymena* contains at least 17 distinct microtubule systems, comparable to the number found in an entire multicellular organism. Yet, *T. thermophila* has only a single α-tubulin gene and two β-tubulin genes that encode the same β-tubulin protein (1). Thus, *T. thermophila* microtubules are composed of a single type of tubulin heterodimer. Nonetheless, multiple isoforms of both tubulins are present (4), which must be produced by secondary modifications.

We show here that a mutation in the β-tubulin gene conferring resistance to microtubule-depolymerizing drugs and hypersensitivity to taxol in *Chlamydomonas* (5) confers a similar phenotype on *T. thermophila*. The mutated *T. thermophila* β-tubulin gene is targeted to the homologous locus and not to the other β-tubulin gene, whose coding region is 96.5% identical. The mutant gene can completely replace the endogenous gene. Taxol-sensitive mutant cells can be retransformed with a wild-type gene to taxol resistance. It will now be possible to introduce tubulins lacking modification sites to study the functions of those modifications *in vivo*.

MATERIALS AND METHODS

Strains, Culture, and Drug Treatments. *T. thermophila* strains CU428.1-Mpr/Mpr, Pm^+/Pm^+ [6-methylpurine (mp)-sensitive, paromomycin (pm)-sensitive, VII] and B2086 Mpr^+/Mpr^+, Pm^+/Pm^+ (mp-sensitive, pm-sensitive, II) were provided by P. J. Bruns (Cornell University, Ithaca, NY). Strain D5H8-4 [Mpr/Mpr^+, Pm^+/Pm^+ (mp-resistant, pm-resistant, mating type unknown)] is an F_1 progeny of CU428.1 and B2086 electrotransformed (6) to paromomycin resistance with the pD5H8 vector. Cells were grown in SPP (7) at 30°C with shaking.

The following stock solutions of drugs were used: paromomycin (Humatin, Parke–Davis), 100 mg/ml in water; 100 mM oryzalin (gift from D. Pennock, Miami University, Oxford, OH) in dimethyl sulfoxide; 20 mM taxol (Sigma) in dimethyl sulfoxide; 100 mM vinblastine sulfate (Sigma) in dimethyl sulfoxide; and 10 mM pronamide (propyzamide, Chem Service, West Chester, PA) in dimethyl sulfoxide. Colchicine (Sigma) was used directly as a powder.

Vectors and Transformation. pBTU1 (Fig. 1) is a pBluescript KS(+) plasmid (Stratagene) containing the β-tubulin 1 (BTU1) gene of *T. thermophila* (1). $pBTU1M^{350}$ is a derivative in which the lysine codon AAT at position 350 was changed to ATG, encoding methionine. pBTU1SC1 is pBTU1 containing a *Sac* I site created by the silent replacement of the GCC at codon 283 with GCT. Modifications were performed by site-directed mutagenesis (8). pD5H8 is an rDNA vector (9) which efficiently transforms *Tetrahymena* to paromomycin resistance by electroporation of conjugants (6). pD5H8 or pH4T2, another high-frequency *Tetrahymena* transformation vector conferring paromomycin resistance (unpublished observations), was used as a cotransforming vector.

Two transformation methods were used. Conjugants were transformed by electroporation (6). The $pBTU1M^{350}$ insert was released by cleavage with *Xba* I and *Hin*dIII restriction endonuclease. Fifty micrograms of cleaved $pBTU1M^{350}$ and 15 μg of uncut pD5H8 or pH4T2 DNA were used for each electroporation. Electroporated cells were resuspended in 20 or 40 ml of SPP medium 1 min after the pulse. Cells were

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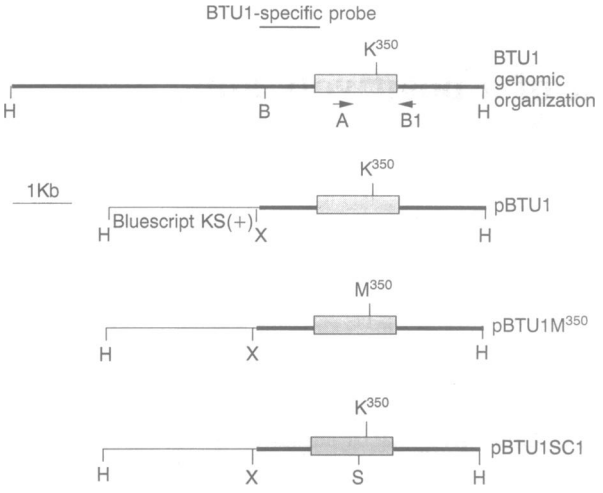

FIG. 1. Genomic structure of the *T. thermophila* BTU1 gene and the plasmid vectors used for gene replacement. Shaded boxes represent the coding sequence of the β-tubulin gene. Arrows indicate positions of the oligonucleotide primers used for PCR amplification. Restriction sites: H, *Hin*dIII; B, *Bgl* II; X, *Xba* I; S, *Sac* I. K^{350} and M^{350} indicate lysine or methionine at position 350 of the β-tubulin gene.

plated on eight or sixteen 96-well microtiter plates (25 μl of cells plus 175 μl of SPP medium per well). Paromomycin was added 18–20 hr later to a final concentration of 100 μg/ml. After 3–4 days, resistant clones were plated in SPP containing 30 μM oryzalin or vinblastine. At the dilution used to select tubulin gene transformants, every well usually contained paromomycin-resistant transformants. The actual transformation frequency was determined by plating some cells at higher dilution and calculated with the Poisson distribution (10).

In the re-replacement experiment, oryzalin-resistant, taxol-sensitive cells were retransformed with linearized pBTU1SC1 by macronuclear microinjection (11).

Extraction and Analysis of Total DNA from *Tetrahymena*. To extract total *Tetrahymena* DNA (M. Zillmann, personal communication), 25 ml of a stationary-phase culture was concentrated to 0.5 ml and combined with 3.5 ml of 42% (wt/vol) urea/0.35 M NaCl/10 mM Tris·HCl, pH 7.4/10 mM EDTA/1% SDS. The solution was gently shaken until homogeneous and then was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol) and once with chloroform/isoamyl alcohol (24:1). One milliliter of 5 M NaCl was added to about 3 ml of aqueous phase and DNA was precipitated with an equal volume of isopropyl alcohol. The DNA was spooled on a glass rod, washed with 70% (vol/vol) ethanol, and dried. The sample was suspended in 600 μl of 10 mM Tris·HCl/1 mM EDTA, pH 8, plus 6 μl of RNase A (10 mg/ml) at 55°C overnight and then centrifuged at 31,000 × *g* for 45 min.

DNA was digested and blotted as described (1). Three probes were used. The oligodeoxynucleotide 5′-AACAACATCATGTCTTCCATT-3′, representing the portion of BTU1 surrounding codon 350 and containing the mutant nucleotide (underlined), was end labeled with [γ-^{32}P]ATP (12). The 1.1-kb *Kpn* I–*Xba* I fragment of pBTU1 containing the 5′ flanking region was used as a BTU1-specific probe. This fragment contains about 100 bp of coding sequence and therefore also recognizes a short fragment of the BTU2 gene (1). As a general β-tubulin probe we used a *Kpn* I–*Eco*RI fragment of a *Tetrahymena pyriformis* BTT1 gene coding sequence (13). Plasmid DNA fragments were labeled by random priming with [α-^{32}P]dATP (12).

Analysis of β-Tubulin Gene Expression by PCR of cDNA. RNA was extracted from growing cells (14). The first strand of cDNA was synthesized by using random hexamer primers (15) and an RNase H-negative Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) according to the manufacturer's protocol. The cDNA was amplified by PCR using primer A (5′-CCGTCCATCAATTGGT-3′), located 563–579 bp downstream from the ATG initiation codon in both the BTU1 and BTU2 genes, and primer B1 (5′-GTTAGCTGACCGATTCAGTTCG-3′) located close to the TGA stop codon in the 3′ flanking region of BTU1 (Fig. 1). PCR amplifications used *Taq* polymerase (Promega) in an Ericomp Twinblock thermal cycler (Ericomp, San Diego). PCR products were digested with *Sac* I and analyzed by electrophoresis in a 1% agarose gel in 40 mM Tris acetate/1 mM EDTA, pH 8.0.

RESULTS

The Mutated BTU1 Gene Transforms *Tetrahymena*. The *col^{r}15* mutant in *Chlamydomonas* is resistant to a variety of antitubulin drugs, including oryzalin, colchicine, and vinblastine, and is hypersensitive to taxol. This mutant has an altered β-tubulin gene in which the lysine residue at position 350 had been mutated to methionine (16). We modified the cloned *T. thermophila* BTU1 gene *in vitro* by site-directed mutagenesis to create the mutation corresponding to *col^{r}15*. The vector containing this mutant gene (pBTU1M^{350}) and either pD5H8 or pH4T2, which confer paromomycin resistance, were introduced into *Tetrahymena* conjugants by electroporation. Cells were selected for paromomycin resistance and then screened for oryzalin or vinblastine resistance.

Ten clones resistant to 30 μM oryzalin or vinblastine were obtained in three experiments (Table 1, Exps. 1–3). Control strains (wild-type CU428 or D5H8-4 cells transformed to paromomycin resistance by pD5H8) were inhibited by 30 μM oryzalin or vinblastine and grew in medium with 20 μM taxol at 30°C. Seven putative tubulin gene transformants were both more resistant to oryzalin or vinblastine and more sensitive to taxol than controls. The remaining three clones were resistant to oryzalin or vinblastine but grew like wild-type controls in taxol. Similarly adapted or spontaneously mutated clones were obtained in a control transformation with only the cotransforming vector (Table 1, Exp. 4). They were not studied further.

Fragment-Mediated Transformation Results in Gene Replacement. Southern blots were used to determine whether the resistant strains were BTU1M^{350} transformants. *Hin*dIII digestion of wild-type genomic DNA yields two fragments of 8 kb and 3 kb (Fig. 2*A*) from the BTU1 and BTU2 genes, respectively (1). At low stringency, an oligonucleotide including mutated codon 350 recognized both BTU1 and BTU2 in all putative transformants, in control strains, and in all wild-type and mutated plasmid templates (Fig. 2*B*). When the same blot was subsequently washed at higher temperature, the probe remained hybridized only to the mutant gene among the plasmid controls and only to the BTU1 gene in 7 of 10 putative transformant DNAs (6 of 8 shown in Fig. 2*C*). The probe did not hybridize to DNAs from control strains under these conditions. All 7 clones in which hybridization was detected were both oryzalin- or vinblastine-resistant and taxol-hypersensitive. The 3 remaining clones, which were only oryzalin-resistant, did not contain the mutated gene. Thus, selection for the dual phenotype of oryzalin or vinblastine resistance and taxol hypersensitivity reproducibly yielded clones transformed by the BTU1M^{350} gene.

The restriction pattern of tubulin genes in the transformants is unchanged from that of wild-type cells. One of the *Hin*dIII sites flanking the BTU1 locus lies outside of the cloned

Table 1. β-Tubulin gene replacement by electrotransformation

Exp.	Replacement vector	Cotransforming vector	pm-res clones*	ory- or vb-res/tax-res clones†	ory- or vb-res/tax-sen clones‡	Gene replacement efficiency§
1	BTU1M^{350}	pD5H8	5,314	0 (0)	2 (2)	0.04
2	BTU1M^{350}	pD5H8	9,860	2 (0)	4 (4)	0.04
3	BTU1M^{350}	pH4T2	2,006	2 (0)	1 (1)	0.05
4	—	pD5H8	41,792	12 (na)	0 (na)	na

Conjugating cells were electroporated 10 hr after mixing and plated at about 4700 (Exps. 1 and 2) or 2350 (Exp. 3) treated cells per well. Eighteen micrograms of cotransforming vector and 50 μg of linear pBTU1M^{350} were used for each electroporation. Eighteen to 20 hr later paromomycin (120 μg/ml) was added. Paromomycin-resistant clones were grown to saturation and replica transferred on medium containing 30 μM oryzalin (Exps. 1, 2, and 4) or vinblastine (Exp. 3). na, Not applicable.

*Calculated number of primary clones resistant to paromomycin (120 μg/ml).

†Number of clones both resistant to 30 μM oryzalin or vinblastine and resistant to 20 μM taxol. The number of gene replacement transformants identified by Southern blotting and with mutant oligonucleotide hybridization is given in parentheses.

‡Number of clones resistant to 30 μM oryzalin or vinblastine and sensitive to 20 μM taxol. The number of gene replacement transformants identified by Southern blotting and with mutant oligonucleotide hybridization is given in parentheses.

§Percent of primary paromomycin-resistant clones in which replacement of BTU1 gene occurred.

fragment in pBTU1M^{350} (Fig. 1). Conservation of the restriction pattern and hybridization of the mutant oligonucleotide only to the BTUI gene in transformants argues that sequence replacement occurred specifically at the BTU1 locus. Isoelectric focusing gels (17, 18) of ciliary proteins (19) showed that transformants synthesized large amounts of a more acidic β-tubulin isotype, consistent with the replacement of lysine by methionine in BTU1M^{350} (data not shown).

Clonal Analysis Indicates That Gene Replacement Is Complete in Some Transformed Lines. The *Tetrahymena* macronucleus is polyploid, with most genes present in about 45 copies in G_1 phase (see ref. 20 for review). Thus, only a fraction of the BTU1 genes in the macronucleus might be replaced by the mutant sequence in transformants. The macronucleus divides amitotically and alleles segregate randomly at each division (21, 22). As a result, the vegetative progeny of initially heterozygous clones eventually become homozygous, a process known as phenotypic assortment. To determine whether transformants retained any copies of the wild-type BTU1 gene, transformed clones were grown in drug-free medium for 25–30 generations and then tested for taxol resistance. If transformant progeny contain any copies of the wild-type BTU1 gene, then upon release from drug pressure some clonal descendants should revert to a wild-type phenotype and become taxol-resistant through random assortment of alleles. About 10^5 cells from each of five transformed lines were analyzed twice, 10 generations apart. Two clones, OA1 and OA5, did not yield any taxol-resistant assortees. Three yielded low numbers (0.07–8% of cells analyzed) of taxol-resistant clones. Assortees were oryzalin-sensitive, as expected. Thus, some transformants clearly still contained wild-type copies of the BTU1 gene. Since OA1 and OA5 did not yield any taxol-resistant assortees, these clones are very likely complete gene-replacement transformants.

The OA1 and OA5 clones were used for a more detailed study of the phenotype mediated by BTU1M^{350}. Transformants were more resistant to oryzalin, vinblastine, and colchicine and were hypersensitive to taxol. Unlike *Chlamydomonas col*^{r}*15* mutants, the transformed *Tetrahymena* clones were not more resistant to pronamide (data not shown). The range of concentrations in which mutant cells grow and wild-type cells are completely inhibited is narrow. The best conditions for selection at 30°C are 30–40 μM oryzalin, 8–10 mM colchicine, and 20–50 μM vinblastine, concentrations only 2- to 2.5-fold higher than those at which wild-type cells grow. Even though this difference is small, the phenotypes associated with the expression of the mutant tubulin are reproducibly selectable.

The BTU1M^{350} Gene Can Be Used for Both Positive and Negative Selection. BTU1 replacement results in a dual phenotype: multiple antitubulin-drug resistance and taxol hypersensitivity. Thus, it should be possible to replace the mutant gene with the wild-type sequence by selecting for taxol resistance. To perform re-replacement of the mutant sequence with the wild-type one, it is necessary to transform the macronucleus in vegetative cells. The only efficient method for transforming the mature macronucleus is by microinjection (9, 11, 23, 24). We microinjected OA1 cells with the pBTU1SC1 plasmid, which contains a wild-type BTU1 gene marked by a *Sac* I site created by a silent substitution. One hundred twenty-six microinjected clones survived and 6 taxol-resistant clones were selected (designated TR1–6). No resistant clones were isolated from 96 noninjected subclones of OA1. As expected, the TR clones were not only resistant to taxol but were also sensitive to oryzalin.

Total DNA extracted from the TR clones digested with *Hin*dIII and *Sac* I was blotted and probed with a fragment of pBTU1 containing the 5′ flanking region of BTU1 and about 100 bp of coding sequence (Fig. 1). In control strains (Fig. 3), the strongly hybridizing band at 8 kb and the faint band at 3 kb correspond to BTU1 and BTU2, respectively. In all transformants the major band migrates at about 6 kb, consistent with the presence of the *Sac* I site 2 kb from one of the *Hin*dIII sites of BTU1 (see Fig. 1). Thus, all of the TR transformants contained the pBTU1SC1 sequence. They also contained a faint band comigrating with the endogenous fragment of 8 kb. This band most likely contains the unchanged micronuclear BTU1 gene but could also contain some nonreplaced macronuclear copies of BTU1M^{350}. Quantitative analysis of the radioactivity of all bands with a PhosphorImager (Molecular Dynamics) indicated that complete or nearly complete replacement occurred in all transformants (data not shown).

To obtain complete re-replacement, the TR clones were grown in taxol-containing medium for about 30 generations to enrich for the taxol-sensitive BTU1M^{350} gene. The cells were then grown for another 20 generations in drug-free medium and selected for vinblastine resistance. No resistant clones were obtained for TR1, -3, -4, -5, and -6 and only a few resistant clones grew in the TR2 line. These results strongly suggest that complete gene replacement occurred in most of the TR transformants.

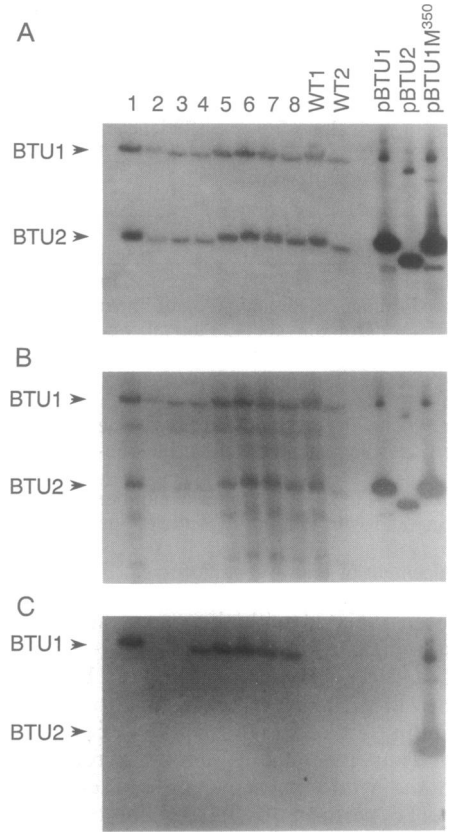

FIG. 2. Southern analysis of DNA extracted from the oryzalin-resistant clones. Total DNA was digested with *Hin*dIII restriction endonuclease, and the fragments were separated in an agarose gel and transferred onto a nylon filter. To identify the two *Tetrahymena* β-tubulin genes (BTU1 and BTU2), the blot was probed with a general β-tubulin coding sequence probe (*A*). A radiolabeled oligomer corresponding to the sequence surrounding codon 350 in the β-tubulin gene and containing the mutant nucleotide was also used as a probe (*B* and *C*). The blot was hybridized, washed at 40°C, and exposed (*B*), washed again at 58°C and reexposed (*C*). The data presented in *A* were obtained by reprobing the same blot shown in *B* and *C* after removal of all radioactive material. Plasmids used as controls were cut with restriction enzymes to release the 3.7-kb BTU1 and 3-kb BTU2 inserts. Note that the plasmid DNA migrates faster than the total genomic DNA, so the BTU2 bands in the total DNA and plasmid samples are not exactly matched. This difference in mobility is frequently observed in the samples of total DNA extracted from the mucus-secreting strains used in this study, possibly due to the presence of contaminating polysaccharides. Lanes 1–8, DNA from putative transformants (clones 1 and 4–8 are oryzalin-resistant and taxol-sensitive; clones 2 and 3 are oryzalin-resistant and taxol-resistant); lanes WT1 and WT2, DNA from two wild-type (nontransformed) lines; lanes pBTU1, pBTU2, and pBTU1M^{350}, plasmid control templates. Under nonstringent conditions the mutant probe hybridized to all plasmid templates and to bands corresponding to both BTU1 and BTU2 genes in all clones analyzed. Under stringent conditions the probe recognized only the mutant plasmid template and the BTU1 gene in all oryzalin-resistant and taxol-sensitive clones.

To confirm the results of the assortment tests, expression of the BTU1 gene was analyzed by taking advantage of the presence of the *Sac* I restriction site in the BTU1SC1 gene. cDNA was synthesized from RNA extracted from the OA1 clone and the TR1 transformant and was amplified by PCR using two primers flanking the *Sac* I site in BTU1SC1 (Fig. 1). PCR products were then digested with *Sac* I. A single product corresponding to the 793-bp full-length fragment of the BTU1 gene was detected in the OA1 host RNA (Fig. 4). In contrast, two smaller fragments were detected in TR1 RNA. No full-size fragment was observed in the products of

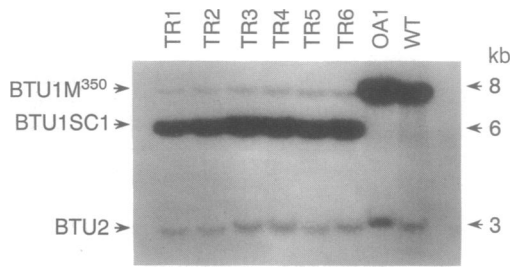

FIG. 3. Southern blot analysis of total DNA of taxol-resistant TR transformants. OA1 taxol-sensitive cells were transformed to taxol resistance by microinjection with the pBTU1SC1 vector. Total DNA was extracted from the transformants and digested with *Hin*dIII and *Sac* I restriction endonucleases. Fragments were separated in an agarose gel, transferred onto a nylon filter, and probed with the radiolabeled BTU1-specific probe containing a small amount of coding sequence. Thus, this probe also cross-hybridized to the small fragment of the BTU2 gene. TR1–6, taxol-resistant transformants; OA1, host taxol-sensitive strain; WT, wild-type control strain CU428. Numbers at right correspond to sizes of DNA marker fragments. Note that in all TR transformants, the major band corresponding to the BTU1 gene was 2 kb smaller than in OA1 or wild-type cells, indicating the presence of a *Sac* I site, consistent with the transformation by gene replacement with pBTU1SC1.

PCR amplification of TR1 RNA, consistent with the complete replacement of the macronuclear BTU1 genes with the *Sac* I site-containing BTU1SC1. Since micronuclei in vegetative *Tetrahymena* are transcriptionally inert (see ref. 20), no transcripts are expected from the micronuclear copies of the BTU1 gene, which lack the *Sac* I site.

DISCUSSION

We created a selectable marker for β-tubulin gene replacement in *Tetrahymena* corresponding to the *col*^{r}*15* mutation

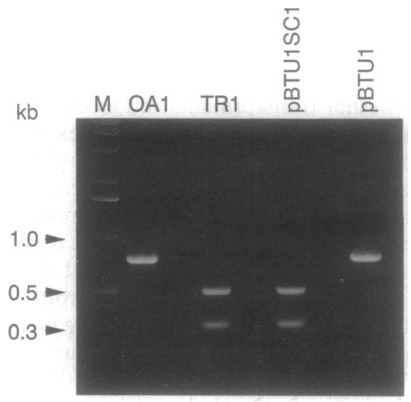

FIG. 4. Analysis of β-tubulin gene expression in transformed cells by PCR amplification. OA1 was transformed to oryzalin resistance and taxol sensitivity by the pBTU1M^{350} vector. TR1 is an OA1 derivative retransformed to taxol resistance and oryzalin sensitivity by microinjection of the pBTU1SC1 vector. Total RNA was extracted from both strains, and cDNA was synthesized. The cDNAs and two control plasmid templates were used for PCR amplification using primers A and B1 (see Fig. 1). The PCR amplification products were digested with an excess of *Sac* I restriction endonuclease, and the fragments were separated in an agarose gel and stained with ethidium bromide. Numbers at left correspond to sizes of DNA marker fragments. In OA1 and the BTU1 plasmid control, only the full-length 793-bp product was detected. In the TR1 transformant and the pBTU1SC1 control, only two smaller products of *Sac* I digestion corresponding to 285 bp and 508 bp were detected, and no full-length product was observed. These results are consistent with the complete replacement of the BTU1 gene in the TR1 transformant with the BTU1SC1 vector containing the *Sac* I restriction site.

described in *Chlamydomonas* (16). Electrotransformation of conjugating *Tetrahymena* (6) was used to replace the endogenous BTU1 gene, one of two coexpressed β-tubulin genes, with this mutated gene. We have also used electroporation of conjugants to successfully replace nine other genes (unpublished observations). Thus, electrotransformation of conjugants with linear DNA fragments is a general method for gene replacement in *Tetrahymena* and should substitute for microinjection-mediated gene replacement (9, 24) in most cases.

BTU1M^{350} transformants display the phenotype expected for the mutation corresponding to *col'15* in *Chlamydomonas*. Both are more resistant to oryzalin, vinblastine, and colchicine and are more sensitive to taxol. As suggested (5), such a phenotype probably reflects an increase in microtubule stability. The similarity of this mutation in *Chlamydomonas* and *Tetrahymena* argues for a critical role of lysine-350 in the dynamic stability of microtubules.

Compared with other selectable markers available in *Tetrahymena*, the BTU1M^{350} gene offers only a modest, 2- to 3-fold increase in drug resistance over wild-type cells, compared with up to a 1000-fold increase for the H4-I-neo paromomycin-resistance gene (24), and an 80-fold increase for the rpl29 cycloheximide-resistance gene (9). The relative weakness of the resistant phenotype conferred by the β-tubulin marker described here may be due to the presence of two coexpressed β-tubulin genes in *Tetrahymena*, only one of which (BTU1) was explored in this study.

As shown here, gene targeting in *Tetrahymena* is highly locus-specific. The mutant BTU1 gene replaced the identical gene in all cases and did not integrate into the highly similar BTU2 locus. The specificity of gene targeting combined with the ease of electroporation and the recent development of a selectable heterologous drug (neomycin) resistance marker (24) should now enable the replacement and knockout of any cloned *Tetrahymena* gene.

Several lines of evidence suggest that complete replacement of the endogenous *Tetrahymena* gene has been achieved. In the original, electroporation-mediated transformants, two clones failed to yield revertants by phenotypic assortment, even when large numbers of cells were tested after long periods of nonselective growth. When these cells were retransformed with a wild-type BTU1 gene containing a unique restriction site created *in vitro*, the fraction of genes lacking the unique site was that expected from micronuclear versions, and transcripts lacking the site were not detectable. Perhaps the most compelling evidence that the original (electroporation-mediated) replacement was complete is that all of the taxol-resistant, microinjected, re-replacement cells contained the injected gene with the unique restriction site. Microinjection-mediated transformation is probably a rare event in which only one or very few of the multiple endogenous macronuclear genes are replaced by the microinjected gene. Since we were able to select taxol-resistant cells expressing the wild-type product of the BTU1 gene after microinjection, we should have been able to select taxol-resistant revertants of the original transformed lines if they still contained a wild-type gene. These lines *never* yielded a taxol-resistant clone, arguing strongly that they did not contain even a single wild-type gene—i.e., that the electroporation-mediated gene replacement was complete.

In conclusion, we have created a selectable marker for β-tubulin gene replacement in *T. thermophila*. The mutant β-tubulin gene confers multiple antitubulin-drug resistance and taxol hypersensitivity and can, in turn, be replaced with the wild-type gene. Thus, mutations can be expressed by using an otherwise unchanged wild-type tubulin gene, avoiding potential interactions between the mutation being studied and a marker mutation. This approach opens the way to study the functions of β-tubulin isoforms, β-tubulin molecular domains, and β-tubulin secondary modification in an organism in which tubulin is assembled into multiple functionally distinct microtubular complexes.

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