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Identification of the Gene Encoding the Tryptophan Synthase β-Subunit from *Chlamydomonas reinhardtii*[1]

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We report the isolation of a *Chlamydomonas reinhardtii* cDNA that encodes the β-subunit of tryptophan synthase (TSB). This cDNA was cloned by functional complementation of a *trp*-operon-deleted strain of *Escherichia coli*. Hybridization analysis indicated that the gene exists in a single copy. The predicted amino acid sequence showed the greatest identity to TSB polypeptides from other photosynthetic organisms. With the goal of identifying mutations in the gene encoding this enzyme, we isolated 11 recessive and 1 dominant single-gene mutation that conferred resistance to 5-fluoroindole. These mutations fell into three complementation groups, *MAA2*, *MAA7*, and *TAR1*. In vitro assays showed that mutations at each of these loci affected TSB activity. Restriction fragment-length polymorphism analysis suggested that *MAA7* encodes TSB. *MAA2* and *TAR1* may act to regulate the activity of *MAA7* or its protein product.

In the unicellular green alga *Chlamydomonas reinhardtii*, there is a paucity of auxotrophic mutations. Classic genetic screens have led to the isolation of only Arg-requiring strains (for review, see Harris, 1989). An attempt to isolate Trp auxotrophs on the basis of resistance to the Trp precursor analog 5-MA was unsuccessful, although strains with altered Trp biosynthetic enzyme activity were recovered (Dutcher et al., 1992). The failure to isolate amino acid auxotrophs has been attributed to the inability to effectively import amino acids other than Arg (Kirk and Kirk, 1978).

Given these observations, we reasoned that an alternative strategy for investigating amino acid metabolism in *C. reinhardtii* would be to isolate the DNA encoding a biosynthetic enzyme that could be used to characterize restriction fragment-length polymorphisms in mutant strains that are resistant to amino acid analogs. We chose to study TSB. Trp synthase performs two enzymatic functions and is composed of two subunits, α and β, which together catalyze the conversion of indole-3-glycerol phosphate to Trp. The α-subunit catalyzes the conversion of indole-3-glycerol phosphate to indole and glyceraldehyde-3-phosphate, whereas the β-subunit catalyzes the condensation of Ser and indole into Trp. TSB was particularly attractive because of its conservation in many organisms (Crawford, 1989; Zhao et al., 1994), and the fact that both auxotrophic and nonauxotrophic mutations affecting TSB in *Arabidopsis thaliana* have been isolated on the basis of resistance to the TSB substrate analog 5-FI (Barczak et al., 1995).

Using a *C. reinhardtii* cDNA library, we complemented an *Escherichia coli* strain lacking TSB activity. The complementing cDNA was sequenced and found to be similar to genes encoding TSB from other organisms. The predicted protein product showed highest identity to homologs from other photosynthetic organisms. Hybridization analysis of genomic DNA indicated that TSB is encoded by a single-copy gene in *C. reinhardtii*.

With the goal of identifying mutations in TSB, we examined 14 prototrophic strains that were resistant to 5-FI. These mutations fell into three complementation groups, *MAA2*, *MAA7*, and *TAR1*. Hybridization analysis of wild-type and mutant DNA probed with the TSB-encoding cDNA revealed a restriction fragment-length polymorphism associated with the *maa7-8* mutation. We conclude that *MAA7* is likely to encode TSB.

MATERIALS AND METHODS

Strains and Culture Conditions

Escherichia coli strain JMB9 Δ*trpEA2* ($r^-m^+leu^-thi^-$) (Yanofsky and Horn, 1995), which was a kind gift of Dr. Charles Yanofsky (Stanford University, CA), was used as the host strain for functional identification of a *Chlamydomonas reinhardtii* cDNA encoding TSB. This strain was maintained in 2× yeast extract tryptone liquid or on Luria-Bertani solid medium (Sambrook et al., 1989). The medium used for selection of Trp prototrophs (minimal indole medium) contained the E salt mixture of Vogel and Bonner (1956) supplemented with indole, biotin, thiamine, Glc, acid-hydrolyzed casein (Zhao et al., 1994), and Leu (40 g mL^{-1}).

The *C. reinhardtii* wild-type strains that we used were 137c mating-type plus (CC125) and mating-type minus

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Abbreviations: 5-FI, 5-fluoroindole; 5-MA, 5-methylanthranilate; 5-MT, 5-methyltryptophan; 6-MA, 6-methylanthranilate; TSB, β-subunit of Trp synthase.

(CC124). Unless otherwise stated, cells cultured in liquid were grown in medium I of Sager and Granick (1953) supplemented with 8 mM sodium acetate (Holmes and Dutcher, 1989) under constant illumination (150 μmol photons m^{-2} s^{-1}) at 21°C. Growth on solid medium took place at 21°C (for most purposes) or at 25°C (when scoring drug resistance) under constant illumination (150 μmol photons m^{-2} s^{-1}).

Isolation of a TSB-Encoding cDNA from *C. reinhardtii*

A wild-type *C. reinhardtii* cDNA library (a generous gift of Dr. Andy Wang, Iowa State University, Ames) in the vector λZAP II (Stratagene) was converted into a phagemid library using the "mass excision of pBluescript from a lambda ZAP library" protocol from Life Technologies. The phagemids were converted into a double-stranded plasmid library using the host strain XLOLR (Stratagene) according to the supplier's instructions. The final isolation of the plasmid library was performed using a Plasmid Maxi Kit (Qiagen, Chatsworth, CA).

This plasmid library was transformed into JMB9 Δ*trpEA2*, which lacks the entire coding region of the *trp* operon (Yanofsky and Horn, 1995). The cells were plated onto minimal indole medium and incubated at 37°C. Plasmids were isolated from trp^+ transformants and sequenced at the DNA Sequencing Facility at Iowa State University. A single plasmid, pCU-100, was chosen for further study.

Computer Analysis of cDNA

The sequence of the *C. reinhardtii* cDNA encoding TSB was compared with DNA and protein sequence databases using the BLASTN (Altschul et al., 1990) and BLASTX (Altschul et al., 1990; Gish and States, 1993) programs, respectively. The predicted translation product of the pCU-100 insert was compared with protein sequence databases using the BLASTP program (Altschul et al., 1990). An alignment of 23 TSB protein sequences obtained from GenBank (Benson et al., 1997) was made using the PILEUP (Devereux et al., 1984) or CLUSTAL-W (Thompson et al., 1994) programs. Phylogenetic trees were inferred by Fitch-Margoliash analysis using a Dayhoff PAM250 substitution matrix with PROTDIST, NEIGHBOR, and FITCH programs from the PHYLIP package, version 3.5 (Felsenstein, 1993, 1996). Unweighted parsimony trees were inferred using the PROTPARS program from the PHYLIP package (Felsenstein, 1993). Trees generated with different alignment programs or with UGMPA, neighbor-joining, and parsimony methods were qualitatively similar.

DNA-Hybridization Analysis

Total *C. reinhardtii* DNA was isolated as described by Newman et al. (1990). Conditions for DNA blotting and hybridization of pCU-100 DNA to genomic DNA were performed as described by Johnson and Dutcher (1991), except that the hybridization temperature was 55°C. Filters were washed in 15 mM Na citrate, 150 mM NaCl, pH 7.0, 0.2% SDS once at room temperature (5 min) and twice at 63°C (20 min each wash). The probe was ^{32}P-labeled using the Multiprime DNA Labeling System (Amersham).

Genetic Analyses

Standard techniques used to determine segregation of genetic markers were performed essentially as described by Harris (1989). Diploid strains were constructed by mating *nit2-1 AC17* strains to *NIT2 ac17-1* strains and selecting for growth on solid medium lacking acetate and containing 2 mM $NaNO_3$ as the sole source of N (Fernández et al., 1989). Because mitotic recombination and chromosome-loss events sometimes confused scoring for analog resistance in complementation tests, at least eight independent diploid strains of a given genotype were examined. For the same reason, potential diploid strains were kept under selective conditions until transfer onto other diagnostic media. The effectiveness of the diploid-strain-selection technique was confirmed by DNA hybridization using the mating-type-specific probe from plasmid pThi4.9 (Ferris, 1995).

Selections for 5-FI Resistance

Each culture used in the selections was derived from an independent single colony. For UV-radiation mutagenesis, 20 cultures of 0.5×10^6 to 1.0×10^6 cells each were plated onto acetate medium and exposed to UV radiation from 20-W sunlamps (model FS20, Westinghouse, Baltimore, MD) for 45 s. This mutagenesis procedure results in approximately 50% lethality. The plates were immediately wrapped in foil and allowed to recover overnight at 21°C. Mutagenized cells were replica plated (RepliPlate, FMC, Inc., Rockland, ME) onto acetate medium supplemented with 25 μM 5-FI (Sigma) and 1.5 mM L-Trp (Sigma) (FIT medium), wrapped in a paper towel to inhibit photodegradation of Trp and 5-FI, and incubated at 25°C under constant illumination (30 μmol photons m^{-2} s^{-1}). For γ-radiation mutagenesis, 35 cultures of 0.5×10^6 to 1.0×10^6 cells were plated onto solid FIT medium. Each was exposed to 10,000 rad, which resulted in approximately 50% lethality, from a ^{137}Cs irradiator (model 143, J.L. Shepherd and Associates, Glendale, CA). Plates of mutagenized cultures were wrapped in a paper towel and incubated under constant illumination (30 μmol photons m^{-2} s^{-1}) at 25°C. Potential 5-FI-resistant colonies were collected for 2 weeks and rescored for growth on FIT medium. Only one 5-FI-resistant colony was collected from a given plate. Each 5-FI-resistant isolate was tested for growth in the presence and in the absence of both Trp and 5-FI.

Subsequent to the isolation of 5-FI-resistant strains, cultures supplemented with light-sensitive compounds were incubated in boxes constructed of one-eighth-inch-thick Yellow-2208 acrylic (Polycast Technology, Inc., Stamford, CT), which filters out wavelengths of light less than 454 nm and inhibits photodegradation of IAA (Stasinopoulos and Hangarter, 1990) and other compounds.

TSB Activity Assays

Cultures for TSB activity assays were grown in acetate medium supplemented with K_2HPO_4 to a final concentra-

tion of 1 mM (Holmes and Dutcher, 1989). Cells were grown to mid-log phase at 21°C and harvested by centrifugation. Cell pellets were resuspended in breaking buffer (Dutcher et al., 1992) to a final concentration of approximately 25% (v/v) and transferred to a 30-mL centrifuge bottle. The resuspended cells were frozen in liquid N_2 and stored at −70°C. Cell extracts were prepared by sonicating thawed cells on ice, followed by passage through a 38.1-mm, 22-gauge syringe needle at 4°C. Debris were removed by centrifugation at 12,000*g* for 10 min at 4°C. Each supernatant was aliquoted into microcentrifuge tubes and stored at −70°C. The protein content of each extract was determined using the Bio-Rad protein assay kit and compared with BSA standards according to the manufacturer's instructions.

TSB activity assays were based on the protocol of Last et al. (1991) with the following modifications. Reactions (7 mL) were preincubated for 10 min at 30°C with all components except indole. At several time points after the addition of indole, 1-mL aliquots were removed and the reaction stopped by mixing with 4 mL of toluene. The indole content of each toluene phase was measured colorimetrically using Ehrlich's reagent prepared as described by Yanofsky (1955), except that the reagent was freshly made for each set of reactions.

One unit of TSB activity in an extract was defined as nanomoles of indole consumed per minute. This value was determined from the absolute value of the slope of the plot of nanomoles of indole versus time. Each calculated activity was normalized according to the amount of protein in each reaction. The slope of a given plot was determined by least-squares analysis. The SE of the specific activity, the weighted mean of multiple specific activities, and the SE of the weighted mean were calculated as described by Bevington and Robinson (1992). Specific activities are presented in units per milligram of total protein.

Drug-Resistance Phenotypes

5-FI-resistant strains were tested for growth on solid acetate medium supplemented with 7 μM 5-FI, 1.6 mM 5-MA, 1.39 mM 6-MA, 1.29 mM 5-MT, 60 μM anisomycin, 2.8 mM canavanine, 4 mM colchicine, 64 μM cycloheximide, or 5 μM oryzalin. Stocks of these compounds were prepared as described by Dutcher et al. (1992) and James and Lefebvre (1989).

RESULTS

Cloning a *TSB* cDNA from *C. reinhardtii*

Because of the high degree of conservation among TSB proteins from different organisms, we sought to identify a TSB-encoding gene from *C. reinhardtii* by rescuing an *E. coli* strain lacking TSB activity. A plasmid library of wild-type *C. reinhardtii* cDNA was transformed into the *trp*-operon-deleted *E. coli* strain JMB9 Δ*trpEA2*. Approximately 10^{10} transformants were plated onto minimal indole medium and grown for 5 d. Five transformants were isolated that survived when restreaked onto selective medium. Plasmids isolated from these transformants were sequenced. Four of the plasmid preparations were not homogeneous, presumably because of some instability of the plasmids. A preparation of the fifth plasmid, pCU-100, was homogeneous and sequenced in its entirety. The 1840-bp cDNA contained a single large open reading frame of 1332 bp. The predicted translation product of 444 amino acids (Fig. 1) displayed strong identity to known and predicted TSB sequences (Fig. 2). The highest scoring matches from a BLASTP search (Altschul et al., 1990) were to TSB sequences from other photosynthetic organisms. For example, the *C. reinhardtii* predicted translation product was 75% identical and 86% similar to the TRP2 precursor protein from maize (*Zea mays*) ($P = 2.0^{-216}$). Identity and similarity to TSB from the cyanobacterium *Synechocystis* sp. were 71 and 83%, respectively ($P = 1.3^{-206}$). Matches to TSB proteins from nonphotosynthetic organisms had slightly lower scores. For example, the predicted *C. reinhardtii* translation product was 61% identical and 76% similar to the TrpB protein from *E. coli* ($P = 6.9^{-152}$).

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   1 GGCACGAGCTCGCCTGGATTCCCGTCCCGTCGCGAGCGCTCGCAGCAGCGCTCGCTCGGT
     A  R  A  R  L  D  S  R  P  V  A  S  A  R  S  S  A  R  S  V
  61 GGCCCCTCGCGTTGCTAGCCGCAAGTCTACTGTTCGCATTGCTGCTGTTGCTGCGCCTGA
     A  P  R  V  A  S  R  K  S  T  V  R  I  A  A  V  A  A  P  E
 121 GCGCCCGATTACCGACTACCAGCGCCCGGACGCAAATGGTCGCTATGGCCAGTTCGGCGG
     R  P  I  T  D  Y  Q  R  P  D  A  N  G  R  Y  G  Q  F  G  G
 181 CAAGTACGTGCCTGAGACCCTGATCCCTGCCCTGGAGCAGCTGGAGAAGGACTACAACGA
     K  Y  V  P  E  T  L  I  P  A  L  E  Q  L  E  K  D  Y  N  E
 241 GGCGATTGCGGACCCTGCGTTTAAGGCGGAGATGGAGGCTATCCTGAAGGACTACGTGGG
     A  I  A  D  P  A  F  K  A  E  M  E  A  I  L  K  D  Y  V  G
 301 TCGCGAGACGCCCCTTTACCACGCCGAGCGCCTGTCGGCGCACTACAAGACCGCGGACGG
     R  E  T  P  L  Y  H  A  E  R  L  S  A  H  Y  K  T  A  D  G
 361 CGGCCACGCTGAGATTTACCTAAAGCGTGAGGACCTGAACCACACTGGCGCGCATAAGAT
     G  H  A  E  I  Y  L  K  R  E  D  L  N  H  T  G  A  H  K  I
 421 CAACAACTCGCTGGGCCAGGCCCTGCTGTGCAAGCGCCTGAACAAGCAGCGCATCATTGC
     N  N  S  L  G  Q  A  L  L  C  K  R  L  N  K  Q  R  I  I  A
 481 GGAGACCGGTGCCGGCCAGCACGGTGTGGCCACGGCCACCATCTGCGCCCGCCTGGGCCT
     E  T  G  A  G  Q  H  G  V  A  T  A  T  I  C  A  R  L  G  L
 541 GAAGTGCATTGTGTACATGGGCGCTAAGGACATGGAGCGCCAGGCGCTGAACGTGTTCCG
     K  C  I  V  Y  M  G  A  K  D  M  E  R  Q  A  L  N  V  F  R
 601 CATGCGCCTGTGCGGTGCGGAGGTCCGCCCCGTGAACAGCGGCACCGCCACGTTGAAGGA
     M  R  L  C  G  A  E  V  R  P  V  N  S  G  T  A  T  L  K  D
 661 TGCCACCAGCGAGGCCATCCGCGATTGGGTCACCAACGTGGAGACCACCCACTACATCCT
     A  T  S  E  A  I  R  D  W  V  T  N  V  E  T  T  H  Y  I  L
 721 GGGGTCGGCCGCTGGCCCCCACCCCTACCCCATGATGGTGCGCGAGTTCCAGTCTGTCAT
     G  S  A  A  G  P  H  P  Y  P  M  M  V  R  E  F  Q  S  V  I
 781 CGGCCGCGAGACCAAGGTGCAGGCGCAGGAGAAGTGGGGCGGCCTGCCCGACATCGTGAT
     G  R  E  T  K  V  Q  A  Q  E  K  W  G  G  L  P  D  I  V  M
 841 GGCGTGCGTGGGCGGCGGCTCCAACGCCATCGGCATCTTCAACGAGTTCATCAACGACAC
     A  C  V  G  G  G  S  N  A  I  G  I  F  N  E  F  I  N  D  T
 901 CTCGGTCCGCCTGATCGGCGTGGAGGCCGGCGGTGAGGGCGTCAACACCACCAAGCACGC
     S  V  R  L  I  G  V  E  A  G  G  E  G  V  N  T  T  K  H  A
 961 CGCCACGCTGACCATGGGCACGCCCGGCGTGCTGCACGGCAGCTACAGCTACCTGCTGCA
     A  T  L  T  M  G  T  P  G  V  L  H  G  S  Y  S  Y  L  L  Q
1021 GGACGATGACGGCCAGATCATTGACCCCCACTCCATCAGCGCCGGCCTGGACTACCCCGG
     D  D  D  G  Q  I  I  D  P  H  S  I  S  A  G  L  D  Y  P  G
1081 CATTGGCCCCGAGCACTCGTTCCTCAAGGACGTGAAGCGCGCAGAGTACTACGCCGTCAC
     I  G  P  E  H  S  F  L  K  D  V  K  R  A  E  Y  Y  A  V  T
1141 GGACGCCGAGGCGCTGGAGGGCTTCCAGCTGCTCAGCAAGTTGGAAGGCATTATCCCCGC
     D  A  E  A  L  E  G  F  Q  L  L  S  K  L  E  G  I  I  P  A
1201 GCTGGAGACCAGCCACGCCATCGCCTACCTGGAGAAGCTGATCCCCACGCTCAAGTCAGG
     L  E  T  S  H  A  I  A  Y  L  E  K  L  I  P  T  L  K  S  G
1261 AACCCGCGTGGTCATTAACTGCTCGGGCCGCGGCGACAAGGACGTCAACAACGCCATGAA
     T  R  V  V  I  N  C  S  G  R  G  D  K  D  V  N  N  A  M  K
1321 GTACATCAACCCTTAAACGACCAGCACTGTGCTTTGACAGACAAGCTCACGCGTGTCGTG
     Y  I  N  P  *
1381 GCGGCGGTATCGGCGCTGTGGATGGGCCCGGGTTTACTGGCTGCGACGCAGCAGCTAGCC
1441 GGCCTGCAGCGCGAAGGAGCGTAGTGTGTACGGCGCGGCTGGATTTTGAAGACGAGGAGC
1501 GAAGGGGCTGATAGTTGGCGCAAGGGTGCTGCCGCGGCGTGCGCAGCGCTGCGTTCCAGT
1561 GGCCCAGTGATTGAAAGGGGAGCGTACGTGACTATGACTTGGGCGTGGGACGAGGCCCGG
1621 TTGGGGCTGAGGCGGATTATGAGTTCGCGTGTGGGAGCGGGAGTGACTGCAGGTTTGTTT
1681 GAGAGCAGAGCGAAGGGCCGCCTTGGTCGCAGTGTGCACTGGGCGTGCTTTGGTGAGACC
1741 GGGGCCGAAGGGAGATGGTTGCAGTACTCACTTTGTTGCTACCAATCGCATGTTGTAATA
1801 ATAATGTTTATAAAAAAAAAAAAAAAAAAAAAAA
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Figure 1. cDNA sequence encoding TSB. The predicted translation product is shown below the DNA sequence. Highlighted amino acids are part of a putative chloroplast transit peptide.

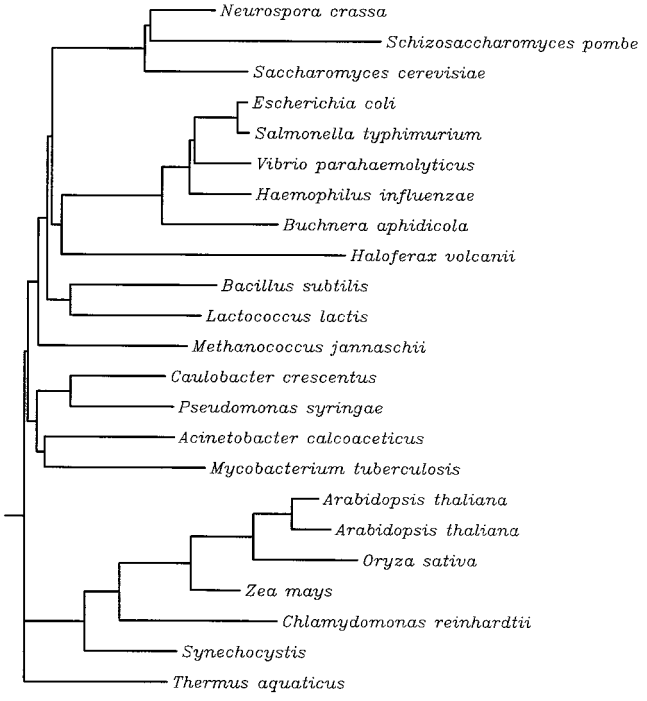

Figure 2. Phylogenetic tree of TSB amino acid sequences from *C. reinhardtii* and 23 other organisms. The tree was generated by the Fitch-Margoliash method (see "Materials and Methods" for details). Included sequences are from *Thermus aquaticus* (GenBank accession no. X58673; Koyama and Furukawa, 1990), *Synechocystis* sp. (GenBank accession no. D64006; Zhao et al., 1994), *Z. mays* TSB1 (GenBank accession no. M76684; Wright et al., 1992), *Oryza sativa* (GenBank accession no. AB003491), Arabidopsis TSB1 (upper) and TSB2 (lower) (GenBank accession nos. M23872 and M81620; Berlyn et al., 1989; Last et al., 1991), *Mycobacterium tuberculosis* (GenBank accession no. Z95554; Philipp et al., 1996), *Acinetobacter calcoaceticus* (GenBank accession no. M34485; Kishan and Hillen, 1990), *Pseudomonas syringae* (GenBank accession no. M95710; Auerbach et al., 1993), *Caulobacter crescentus* (GenBank accession no. M19129; Ross and Winkler, 1988), *Methanococcus jannaschii* (GenBank accession no. U67546; Bult et al., 1996), *Lactococcus lactis* (GenBank accession no. M87483; Bardowski et al., 1992), *Bacillus subtilis* (GenBank accession no. D14069; Henner et al., 1985), *Haloferax volcanii* (GenBank accession no. M36177; Lam et al., 1990), *Buchnera aphidicola* (GenBank accession no. Z21938; Lai et al., 1995), *Haemophilus influenzae* (GenBank accession no. P43760; Fleischmann et al., 1995), *Vibrio parahaemolyticus* (GenBank accession no. X17149; Crawford et al., 1991), *Salmonella typhimurium* (GenBank accession no. J01810; Crawford et al., 1980), *E. coli* (GenBank accession no. V00372; Crawford et al., 1980), *S. cerevisiae* (GenBank accession no. V01342; Zalkin and Yanofsky, 1982), *Schizosaccharomyces pombe* (GenBank accession no. D89113), and *Neurospora crassa* (GenBank accession no. P13228; Burns and Yanofsky, 1989). For *N. crassa* and *S. cerevisiae*, the first 300 amino acids were removed because they encode the α-subunit of Trp synthase as part of a single protein.

The lack of an AUG start codon suggested that the 5′ end of the cDNA was missing from pCU-100. The observation that the truncated cDNA produced a functional protein in *E. coli* indicated that the missing sequence is not essential for function in this bacterium. TSB sequences from other photosynthetic eukaryotes contain chloroplast-localization peptides at their amino terminus (Berlyn et al., 1989; Wright et al., 1992; Zhao and Last, 1995). Stromal-targeting domains have no amino acid consensus, but they are generally enriched in hydroxylated amino acids and deficient in acidic amino acids. The amino terminus of the transit peptide is generally devoid of Gly and Pro and charged amino acids; the middle region is generally enriched in Ser, Thr, Arg, and Lys; and the carboxy terminus contains a possible processing site of Val-X-Ala (Gavel and von Heijne, 1990). It is therefore likely that the amino-terminal domain of *C. reinhardtii* TSB is a chloroplast-localization peptide.

The relationship of *C. reinhardtii* TSB to other TSB enzymes was investigated by constructing phylogenetic trees. The alignment for the tree shown in Figure 2 was made using PILEUP (Devereux et al., 1984) and the phylogeny was constructed using the method of Fitch and Margoliash (1967). We observed clustering of the *C. reinhardtii* TSB with the enzymes from other photosynthetic organisms, including the cyanobacterium *Synechocystis* sp. Trees generated with and without the chloroplast transit peptides of Arabidopsis, maize, rice, and *C. reinhardtii* were qualitatively similar.

Copy Number in *C. reinhardtii*

In *A. thaliana* and maize, the gene encoding TSB exists in two copies (Berlyn et al., 1989; Last et al., 1991; Wright et al., 1992). We used the *C. reinhardtii* cDNA from pCU-100 as the probe of genomic DNA blots to determine the copy number of this gene. When genomic DNA was digested with the restriction enzyme *Sal*I, which does not cut within the cDNA, a single *TSB*-specific hybridization band was found (Fig. 3, lanes 1 and 2). Digests with *Acc*I and *Sst*I (either alone or in a double digest), which cut near the 5′ end of the cDNA, also yielded a single hybridization band (data not shown). Presumably, the sequence 5′ of the restriction sites had insufficient homology to allow hybridization or was contained within a fragment too small to be retained under our experimental conditions. Likewise, when genomic DNA was digested with *Nco*I, a restriction enzyme that cuts once in the middle of the cDNA, two hybridization bands were observed (Fig. 3, lanes 3 and 4). Although we cannot exclude the possibility of a highly divergent copy of the gene, we conclude that a single gene encodes TSB in *C. reinhardtii*.

Isolation of Mutant Strains with Altered TSB Activity

In many Trp-producing organisms, TSB converts the indole analog 5-FI into the toxic Trp analog 5-fluorotryptophan (Miozzari et al., 1977; Barczak et al., 1995; for review, see Somerville, 1983). In *A. thaliana*, plants with defects in TSB activity have been isolated by virtue of resistance to 5-FI (Barczak et al., 1995). With the intention of obtaining mutations in the gene encoding *C. reinhardtii* TSB, we isolated a collection of 5-FI-resistant strains. Five independent 5-FI-resistant strains were isolated from approximately 1.5×10^7 cells mutagenized with UV light, and seven independent strains were isolated from approxi-

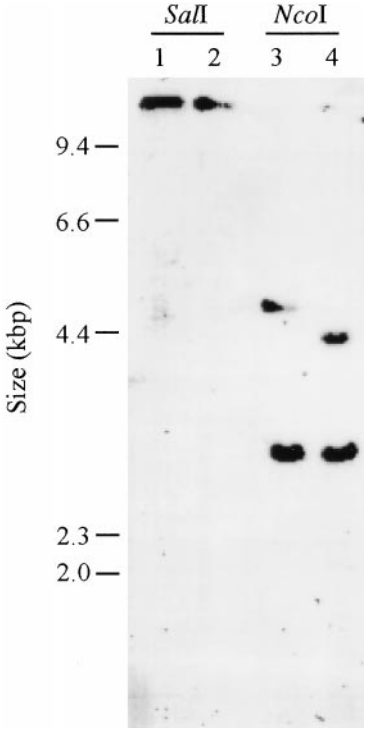

Figure 3. Wild-type and *maa7-8* genomic DNA probed with the TSB-encoding cDNA insert from pCU-100. Genomic DNA from wild-type (lanes 2 and 4) and *maa7-8* (lanes 1 and 3) cultures were digested with the indicated enzymes and probed with the 1.8-kb cDNA. A restriction fragment-length polymorphism is obvious in hybridizations to *Nco*I-digested DNA (lanes 3 and 4).

Table I. *Genetic characterization of 5-FI-resistant strains*

Locus and Allele	Isolation Name	Dominance of 5-FI Resistance	Mapping Data		
			Marker	Linkage group	No. of tetrads[a]
maa2-1	M45[b]	Recessive	*pf15*	III	48:0:5[b]
			nit2-1	III	40:0:24[c]
			maa7-7	III	8:0:0
maa2-8	M24	Recessive	*nit2-1*	III	8:0:10
			maa7-3	III	17:0:3
			maa7-4	III	76:0:6
maa7-2	M21[b]	Recessive	*maa2-1*	III	160:0:11[b]
			maa7-7	III	23:0:0
maa7-3	F4	Recessive	*nit2-1*	III	14:0:9
maa7-4	F5	Recessive	*nit2-1*	III	13:0:8
maa7-5	F9	Recessive	*nit2-1*	III	60:0:47
			maa7-7	III	67:0:0
maa7-6	F18	Recessive	*nit2-1*	III	20:0:11
maa7-7	F21	Recessive	*nit2-1*	III	14:0:15
maa7-8	M22	Recessive	*nit2-1*	III	4:0:3
			maa7-7	III	12:0:0
MAA7-9	M34	Dominant	*nit2-1*	III	5:0:2
			maa2-8	III	47:0:3
			maa7-4	III	130:0:0
tar1-1	M7	Recessive	*tar1-2*	VI	76:0:0
			pf14	VI	18:0:5
tar1-2	M20	Recessive	*pf14*	VI	42:0:17
			act2	VI	27:0:6
tar1-3	M23	Recessive	*tar1-1*	VI	36:0:0
			maa2-8	III	3:3:15
tar1-4	M25	Recessive	*tar1-1*	VI	29:0:0

[a] Ratio of parental ditype: nonparental ditype: tetratype tetrads from the indicated crosses. [b] Data from Dutcher et al. (1992). [c] Combined data from Dutcher et al. (1992) and this study.

mately 2.5×10^7 cells mutagenized with γ radiation (Table I). In an attempt to isolate Trp auxotrophs, the selection medium contained 1.5 mM Trp. However, each resistant strain grew in the absence of exogenous Trp. Incubation at 15, 21, 25, and 30°C had no apparent effect on Trp prototrophy.

Each 5-FI-resistant strain was repeatedly backcrossed to the 137c wild-type strain CC-124. In each case, the mutant strain showed a 2:2 segregation pattern of 5-FI resistance to 5-FI sensitivity, suggesting that a single mutation conferred resistance in a given strain.

Genetic Analysis of 5-FI-Resistance Mutations

Crosses were performed to determine the genetic location of the 5-FI-resistance mutations. Because these mutations might have represented new alleles of previously mapped 5-FI-resistance mutations (Dutcher et al., 1992), each mutation was mapped with respect to *maa2-1*, *maa7-2*, or other loci on linkage group III. As shown in Table I, eight of the mutations were linked to *maa2-1* and *maa7-2*, and map approximately 20 map units from the *NIT2* locus. The other four mutations were found to be linked to each other on linkage group VI (Table I). A genetic map of these loci is shown in Figure 4.

Each mutation was tested for dominance of the 5-FI-resistance phenotype. As shown in Table I, only the mutation in strain M34 was dominant. Complementation tests were performed on the strains with recessive mutations. Failure to complement produced diploid strains that were resistant to 5-FI, and complementation produced diploid strains that were sensitive to 5-FI. All unlinked mutations were found to complement. Of eight recessive mutations on linkage group III, five complemented *maa2-1* but failed to complement *maa7-2*. These mutations have been assigned as alleles of the *MAA7* locus, *maa7-3* through *maa7-8* (Table I). The mutation in strain M24 complemented each

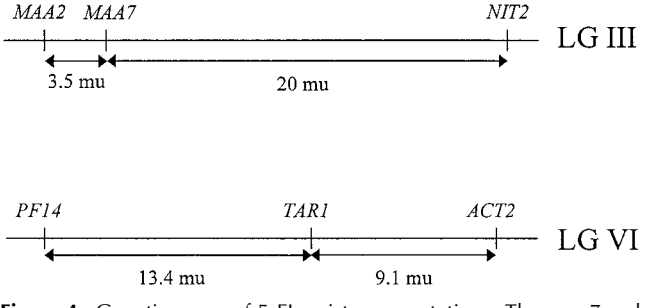

Figure 4. Genetic maps of 5-FI-resistance mutations. The *maa7* and *maa2* mutations map approximately 20 and 23.5 map units (mu) centromere-distal to *NIT2* on linkage group III (top). The *tar1* mutations map between *PF14* and *ACT2* on linkage group VI (bottom). LG, Linkage group.

maa7 allele and failed to complement *maa2-1*. This mutation was designated *maa2-8* (Table I). Because the dominant mutation in strain M34 could not be used in complementation tests, its locus was determined by recombination with known 5-FI-resistance loci. Tetrad data showed that the M34 mutation was inseparable from a *maa7* mutation in 130 tetrads (Table I). In contrast, the mutation was separable from a *maa2* mutation in 50 tetrads (Table I). These data are consistent with the M34 mutation being in the *MAA7* locus, and the mutant allele was named *MAA7-9*.

To determine the relative order of *MAA2* and *MAA7* on linkage group III, three-point crosses using mutations at *MAA2*, *MAA7*, and *NIT2*, a third locus on linkage group III, were performed. The frequency with which various recombinant progeny were recovered (data not shown) was consistent with a gene order of *MAA2 – MAA7 – NIT2* (Fig. 4).

The four tightly linked mutations on linkage group VI were found to belong to a single complementation group at a previously unmapped locus. These mutations have been named as alleles of a new locus, *TAR1* (*T*rp-related *a*nalog *r*esistance) (Table I).

Slow-Growth Phenotypes in 5-FI-Resistant Strains

In addition to its analog-resistance phenotype, the *maa2-1* mutation confers a slow-growth phenotype (Dutcher et al., 1992). Likewise, two of the 5-FI-resistant strains isolated in this study, *maa2-8* and *maa7-3*, displayed a slow-growth phenotype after 9 d of growth. This phenotype was most noticeable in meiotic progeny of crosses between these strains, and a strain without growth defects in which single colonies could be viewed side by side under identical conditions (see Fig. 6). Because each of these strains has a similar plating efficiency, it is likely that the observed growth defects are the result of an increased doubling time. The strain *maa2-1* also had an increased doubling time (Dutcher et al., 1992).

TSB Activity in Wild-Type and 5-FI-Resistant Strains

Crude cell extracts of wild-type and mutant cells were assayed for TSB activity (Fig. 5). In each assay that had measurable TSB activity, the reaction rate was linear with time and protein concentration. Extracts from strains with the *maa2-1* or *maa2-8* mutation had approximately 65 and 75% of wild-type activity, respectively (Fig. 5). Assays of *maa2-1* extracts for anthranilate synthase activity, and combined anthranilate phosphosphoribosyl transferase/phosphoribosyl anthranilate isomerase/indoleglycerol phosphate synthase assays showed no reduction in these enzymatic activities (Dutcher et al., 1992). Taken together, these results indicated that mutations at the *MAA2* locus affect Trp synthase activity specifically. Because the activity of Trp synthase α was not assayed, it is not known whether both Trp synthase α activities were affected.

Extracts from strains with a mutation at the *MAA7* locus had a variety of TSB activities (Fig. 5), ranging from immeasurable activity in *maa7-5* extracts to full wild-type activity in *maa7-3* extracts. Extracts from strain *maa7-1* have been shown to have wild-type levels of anthranilate synthase and anthranilate phosphosphoribosyl transferase/phosphoribosyl anthranilate isomerase/indoleglycerol phosphate synthase activity (Dutcher et al., 1992). Thus, it appears that mutations at the *MAA7* locus specifically affect Trp synthase activity.

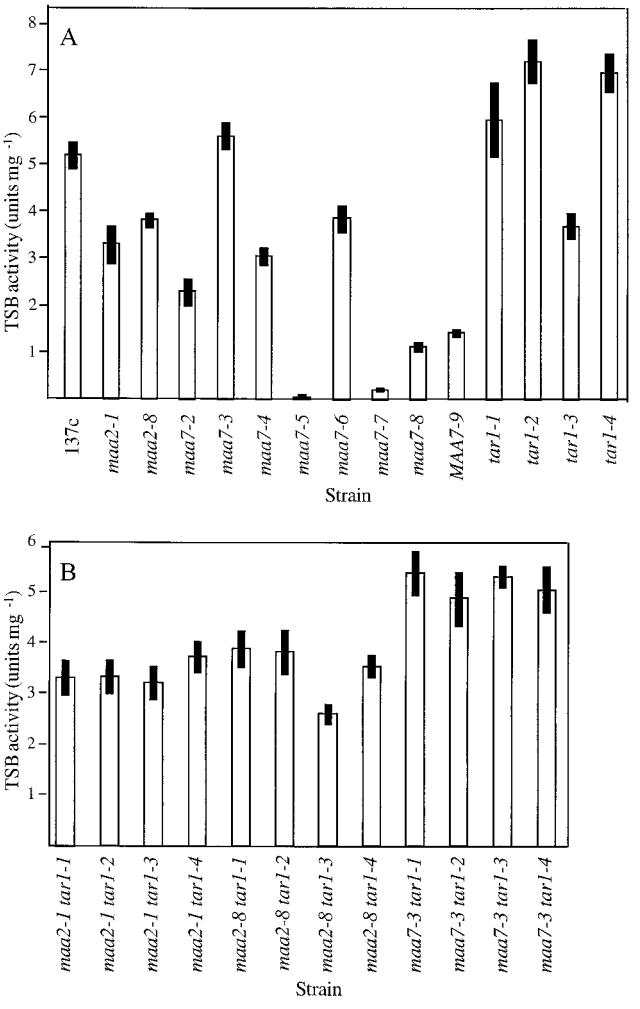

Figure 5. TSB activity of wild-type and 5-FI-resistant strains. Each column represents the weighted mean units of TSB activity per milligram of protein in extracts of the indicated strains. One unit consumes 1 nmol of indole per min. The error bars indicate two SD above and below the mean. A, TSB activity in wild-type and single-mutant strains. B, TSB activity in double-mutant strains.

Alleles of *tar1* were more variable in that extracts from *tar1-2* and *tar1-4* had TSB activity greater than that of wild-type extracts. The *tar1-3* mutation led to reduced TSB activity, approximately 70% of wild-type activity. The *tar1-1* mutation had no significant effect on TSB activity (Fig. 5). Although each assay was performed multiple times, the significance of the increased level is not clear because they are based on crude extracts.

The slow-growth phenotype caused by the mutations *maa2-1*, *maa2-8*, and *maa7-3* is an in vivo effect that may reflect a more severe defect in Trp metabolism. In contrast, the TSB activity assays were performed in vitro, and the observed activities may not accurately reflect in vivo TSB

activity. For example, the *maa7-5* mutation results in undetectable TSB activity in vitro, but strains with this mutation have no growth defect in vivo. We therefore constructed double-mutant strains between *maa2-1*, *maa2-8*, and *maa7-3* and each *tar1* mutation.

Extracts from each double-mutant strain were assayed for TSB activity. As shown in Figure 5, 11 of the double-mutant extracts had the same TSB activity as the corresponding slow-growing single-mutant extract. Extracts of *maa2-8 tar1-3* double-mutant strains had lower TSB activity than either parental single-mutant strain extract. This enhanced reduction of in vitro TSB activity (Fig. 5) was reflected by an enhanced in vivo-growth defect. On solid medium with or without Trp, the double-mutant strain *maa2-8 tar1-3* grew more slowly than either single-mutant strain (Fig. 6). The growth of other double-mutant strains was indistinguishable from that of the parental slow-growing single-mutant strain (data not shown).

The proximity of *maa2* and *maa7* mutations on linkage group III (Fig. 4) precluded the recovery of nonparental ditype tetrads from which double-mutant strains could be unambiguously identified. In crosses of *maa2-8* and *MAA7-9*, however, putative double-mutant strains were identified from tetratype tetrads on the basis of a distinct phenotype. These tetrads consisted of two 5-FI-resistant zygospores (presumed single-mutant strains), one 5-FI-sensitive zygospore (the presumed wild-type recombinant), and one zygospore that died after approximately four rounds of cell division (the presumed double-mutant strain). Thus, the mutations *maa2-8* and *MAA7-9* produce a lethal phenotype when present together.

Other Drug-Resistance Phenotypes

Because mutations at *MAA2*, *MAA7*, and *TAR1* each affect TSB activity in vitro, other means were necessary to determine which of these loci encodes TSB. Mutations in TSB would be expected to display a predictable range of cross-resistance phenotypes (for review, see Sommerville, 1983). Mutations in TSB (a) should not confer resistance to toxins unrelated to Trp biosynthesis, (b) may confer resistance to analogs of other intermediates of Trp biosynthesis, and (c) should not confer resistance to 5-MT, which is an analog of Trp rather than of a biosynthetic intermediate of Trp.

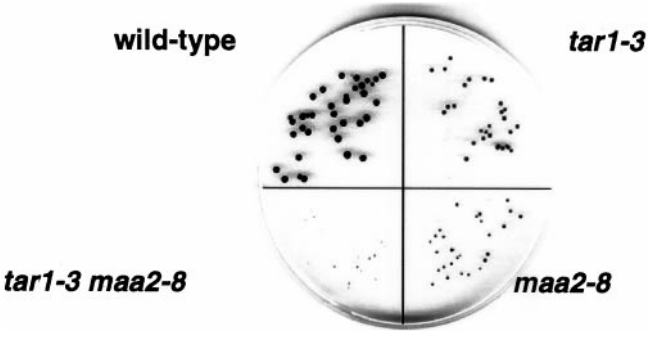

Figure 6. Slow-growth phenotypes associated with 5-FI-resistance mutations. The cells were plated onto acetate medium and grown for 6 d at 21°C. The two single-mutant strains produced smaller colonies than the wild-type strain. However, after 9 d of growth, the *tar1-3* strain produced colonies with a size comparable to wild-type colonies. The colonies from the double-mutant, *tar1-3 maa2-8*, were always smaller than either the single-mutant or wild-type colonies. The same phenotypes were observed in medium supplemented with 1.5 mM L-Trp.

Table II. *Cross-resistance phenotypes of 5-FI-resistant strains*

Strains were spotted onto solid acetate medium supplemented with 7 μM 5-FI, 1.6 mM 5-MA, 1.39 mM 6-MA, or 1.29 mM 5-MT. The presence or absence of growth after 9 d is indicated by a + or −, respectively.

Strain	Growth			
	5-FI	5-MA	6-MA	5-MT
137c	−	−	−	−
maa2-1	+	+	+	+
maa2-8	+	+	+	+
maa7-2	+	+	+	−
maa7-3	+	+	+	−
maa7-4	+	+	+	−
maa7-5	+	−	−	−
maa7-6	+	+	−	−
maa7-7	+	+	+	−
maa7-8	+	+	+	−
MAA7-9	+	+	+	−
tar1-1	+	+	+	+
tar1-2	+	+	+	+
tar1-3	+	+	+	+
tar1-4	+	+	+	+

Each 5-FI-resistant strain was assayed for resistance to a range of toxins unrelated to Trp metabolism (data not shown). Each strain was sensitive to anisomycin (an inhibitor of protein synthesis), canavanine (an analog of Arg), oryzalin (an herbicide that disrupts microtubule-based structures), colchicine (an inhibitor of microtubule polymerization), and cycloheximide (an inhibitor of protein synthesis). Thus, mutations at each locus affected Trp metabolism specifically.

Studies of *A. thaliana* have shown that mutations affecting TSB activity can confer resistance to the anthranilate analogs 5-MA and 6-MA (Last and Fink, 1988; Li and Last, 1996). In *C. reinhardtii* the mutations *maa2-1* and *maa7-2* were isolated based on resistance to 5-MA (Dutcher et al., 1992). We tested each 5-FI-resistant strain for cross-resistance to 5-MA and 6-MA. Strains with any of the *maa2* or *tar1* alleles were fully resistant to 1.6 mM 5-MA and 1.39 mM 6-MA (Table II). Mutations at the *MAA7* locus displayed a range of phenotypes (Table II). Six *maa7* mutations conferred resistance to both 5-MA and 6-MA. In contrast, the *maa7-5* mutation failed to confer resistance to either analog. Strains with the *maa7-6* mutation were resistant to 5-MA and sensitive to 6-MA. Combined with the range of biochemical phenotypes (Fig. 5), these data indicated that a series of nonidentical mutations at the *MAA7* locus was recovered. Resistance to 5-FI, 5-MA, and 6-MA conferred by the *maa2*, *maa7*, and *tar1* mutations may result from reduced or eliminated conversion of the intermediate analog into a Trp analog (Tilby, 1978).

Each strain was tested for resistance to 5-MT. Resistance to 5-MT is presumed to be mediated by an alteration of one or more of the targets of 5-MT. These targets may include enzymes that use Trp as a substrate and proteins for which Trp is an inhibitor or cofactor (for review, see Somerville, 1983). These mechanisms of toxicity suggest that a mutant form of TSB would not confer resistance to 5-MT. As shown in Table II, each *maa2* and *tar1* mutation conferred resistance to 5-MT, whereas no *maa7* mutation conferred resistance. These data are consistent with the hypothesis that *MAA7* encodes TSB. *MAA2* and *TAR1* may encode other products involved in Trp metabolism.

TSB Hybridization Analysis of *maa7-8*

The demonstrated biochemical and drug-resistance phenotypes of *maa7* strains suggested that *MAA7* encodes TSB. To gain additional evidence, we looked for restriction-fragment-length polymorphisms between wild-type and *maa7* cells. We focused on the *maa7-8* allele. Blots of genomic DNA from wild-type and *maa7-8* strains digested with *Acc*I, *Ava*I, *Nco*I, *Sst*I, *Nar*I, and *Sal*I and DNAs were probed with the 1.8-kb cDNA from pCU-100. The first four enzymes have recognition sites within the cDNA, whereas the last two do not. Restriction-fragment-length polymorphisms between the two strains were observed using the restriction enzymes *Nco*I (Fig. 3, lanes 3 and 4), *Ava*I, and *Nar*I, whereas the other enzymes did not produce polymorphisms. The strain-specific hybridization patterns of *Nco*I-digested DNA were observed in DNA isolated from nine random meiotic progeny of a cross of wild-type and *maa7-8* cells (data not shown). Thus, three independent lines of evidence, altered in vitro-TSB activities, drug-resistance phenotypes, and gene-specific genome alterations, suggest that *MAA7* encodes TSB.

DISCUSSION

We report the isolation of a *C. reinhardtii* cDNA that encodes TSB. The lack of a 5′ untranslated region and start codon indicate that the cDNA is incomplete. Despite this, the cDNA complements a *trpB* mutation in *E. coli*, suggesting that the missing sequence is not essential for function in the organism. All but approximately the amino-terminal 46 amino acids encoded by the cDNA showed significant identity to other TSB proteins. It is likely that these 46 amino acids are part of a chloroplast-transit peptide that is present in TSB from other photosynthetic eukaryotes. The presence of a chloroplast-localization sequence at the 5′ end of the cDNA suggests that TSB is a monofunctional polypeptide in *C. reinhardtii*. This finding is consistent with findings in bacteria and plants (Berlyn et al., 1989; Crawford, 1989; Wright et al., 1992). In contrast, Trp synthase α and TSB activities are contained within a single polypeptide in fungi (Zalkin and Yanofsky, 1982; Burns and Yanofsky, 1989), and TSB is part of a pentafunctional Trp biosynthetic polypeptide in *Euglena gracilis* (Hankins and Mills, 1977). A phylogenetic analysis of the predicted translation product and 23 other TSB proteins showed that *C. reinhardtii* TSB is most closely related to TSB enzymes from other photosynthetic organisms. This relationship is maintained even when chloroplast transit peptides are excluded from phylogenetic analyses. Hybridization analysis of *C. reinhardtii* genomic DNA probed with the cDNA from pCU-100 suggested that the gene exists in a single copy. This is based on observing single bands in digests with enzymes that cut once or not at all in the cDNA (Fig. 3). Although both *A. thaliana* and maize have two copies of *TSB* (Berlyn et al., 1989; Last et al., 1991; Wright et al., 1992), *C. reinhardtii* is likely to have only a single gene.

TSB catalyzes the final step of Trp biosynthesis, which is the conversion of Ser and indole into Trp. In the presence of the indole analog 5-FI, TSB also catalyzes the synthesis of the toxic Trp analog 5-fluorotyptophan. Mutations that alter the specificity or activity of TSB could confer resistance to 5-FI. We found that mutations in any of the three loci, *MAA2*, *MAA7*, or *TAR1*, conferred resistance to 5-FI in *C. reinhardtii*. A series of tests was performed to determine whether any of these loci encodes TSB. Each strain was sensitive to a wide variety of toxins unrelated to Trp metabolism, suggesting that the mutations affected Trp metabolism specifically.

One would predict that a subset of *TSB* mutations would confer resistance to analogs of other biosynthetic intermediates of Trp. The anthranilate analogs 5-MA and 6-MA are converted into toxic Trp analogs in many organisms, and mutations in *TSB* can confer resistance to these compounds (for review, see Somerville, 1983; Last and Fink, 1988; Li and Last, 1996). Among the *C. reinhardtii* strains examined, all *maa2* and *tar1* alleles conferred resistance to both 5-MA and 6-MA. The *maa7* mutations examined showed allele-specific variation in cross-resistance phenotypes. This variation was compelling evidence that a series of nonidentical mutations at *MAA7* was recovered.

Because 5-MT is an analog of Trp rather than of a biosynthetic intermediate of Trp, a gene encoding a 5-FI-resistant form of TSB would not be expected to confer resistance to 5-MT. In this study each *maa7* strain was sensitive to 5-MT. In contrast, each *maa2* and *tar1* allele conferred 5-MT resistance. These data suggest that *MAA7* encodes TSB.

The hypothesis that *MAA7* encodes TSB was confirmed by hybridization studies of *TSB* cDNA to genomic DNA. A restriction fragment-length polymorphism cosegregated with the *maa7-8* 5-FI-resistance phenotype in nine meiotic progeny. The likelihood that this cosegregation occurred by chance is less than 0.2%.

The question of the function of *MAA2* and *TAR1* remains. The 5-MT-resistance phenotype associated with mutations at these loci are consistent with the hypothesis that Trp directly or indirectly interacts with the products of these loci. In other organisms, 5-MT toxicity is mediated by incorporation into protein or inhibition of proteins normally regulated by Trp. In at least some bacteria (Pratt and Ho, 1975), fungi (Miozzari et al., 1977), and cultured mammalian tissue (Taub, 1977), part or all of Trp analog toxicity is based on incorporation of these analogs into protein.

In *B. subtilis*, a mutant tryptophanyl-tRNA synthetase confers resistance to 5-MT (Chow and Wong, 1996). In *E. coli*, fluorotryptophans and methyltryptophans also act by false corepression of the *trp* operon and false-feedback

inhibition of anthranilate synthase and Trp-specific deoxy-D-*arabino*-D-heptulosonatephosphate synthase (for review, see Somerville, 1983). False-feedback inhibition of anthranilate synthase is also observed in *S. cerevisiae* (Miozzari et al., 1977) and *A. thaliana* (Li and Last, 1996). In *A. thaliana*, a feedback-resistant form of anthranilate synthase confers resistance to a wide range of analogs of anthranilate and Trp (Li and Last, 1996). If *MAA2* and *TAR1* encode enzymes that are normally feedback inhibited by Trp, the observed alterations in TSB activity would likely be an indirect effect of the mutations. An indirect effect on TSB activity would also be likely if either locus encoded a mutant tryptophanyl-tRNA synthetase or $tRNA^{Trp}$. In addition, Trp synthase α physically interacts with TSB in other organisms, either as independently translated subunits (for review, see Hyde and Miles, 1990; Radwanski et al., 1995) or as a multifunctional polypeptide (Hankins and Mills, 1977; Zalkin and Yanofsky, 1982; Burns and Yanofsky, 1989; Schwarz et al., 1997).

Mutations that affect the structure of Trp synthase α could therefore affect TSB activity. The lethality phenotype of the *maa2-8 MAA7-9* double-mutant strains is consistent with any of these models. Regardless of the nature of the *maa2-8* mutation, the indirect reduction in TSB activity was lethal in combination with the mutant form of TSB encoded by *MAA7-9*. It is worth noting that the *maa7-1* mutation, not included in this study, displayed a conditional lethal phenotype. Strains with this mutation failed to grow at 16°C (Dutcher et al., 1992).

The isolation of a *C. reinhardtii* cDNA encoding wild-type TSB by complementation of an *E. coli* mutation suggests that other cDNAs encoding amino acid biosynthetic enzymes may be similarly obtained. This technique, combined with the ability to isolate and identify prototrophic mutations in an amino acid biosynthetic pathway, has great potential to allow studies of *C. reinhardtii* metabolism that previously have been intractable.

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The accession number for the TSB sequence reported in this article is AF047024.

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