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Sequences Controlling Transcription of the *Chlamydomonas reinhardtii* β_2-Tubulin Gene after Deflagellation and during the Cell Cycle†

JOHN P. DAVIES* AND ARTHUR R. GROSSMAN

Department of Plant Biology, Carnegie Institution of Washington, Stanford, California 94305

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In *Chlamydomonas reinhardtii*, transcripts from the β_2-tubulin gene (*tubB2*), as well as those from other tubulin-encoding genes, accumulate immediately after flagellar excision as well as at a specific time in the cell cycle. Control of *tubB2* transcript accumulation following deflagellation is regulated, at least partially, at the transcriptional level. We have fused the *tubB2* promoter to the arylsulfatase (*ars*) reporter gene, introduced this construct into *C. reinhardtii*, and compared expression of the chimeric gene with that of the endogenous *tubB2* gene. After flagellar excision, transcripts from the *tubB2/ars* chimeric gene accumulate with kinetics similar to those of transcripts from the endogenous *tubB2* gene. The *tubB2/ars* transcripts also accumulate in a cell cycle-specific manner; however, chimeric transcripts are more abundant earlier in the cell cycle than the endogenous *tubB2* transcripts. To elucidate transcriptional control of *tubB2*, we have mutated or removed sequences in the *tubB2* promoter and examined the effect on transcription. The *tubB2* promoter shares features with the promoters of other tubulin-encoding genes; these include a GC-rich region between the TATA box and the transcription initiation site and multiple copies of a 10-bp sequence motif that we call the tub box. The *tubB2* gene contains seven tub box motifs. Changing the GC-rich region to an AT-rich region or removing three of the seven tub box motifs did not significantly affect transcription of the chimeric gene. However, removing four or five tub box motifs prevented increased transcription following deflagellation and diminished cell cycle-regulated transcription from the *tubB2* promoter.

In *Chlamydomonas reinhardtii*, tubulin synthesis increases immediately after deflagellation (28, 34) and during the mitotic phase of the cell cycle (26, 35). Tubulin is required following deflagellation for growth of new flagella and during the cell cycle both for construction of the mitotic spindle apparatus and for regrowth of flagella. The increase in tubulin synthesis coincides with a transient increase in the accumulation of tubulin mRNA (1, 7, 23, 32). After deflagellation, the increased accumulation of the tubulin mRNA is a result of an elevated rate of transcription (3, 20) and probably an increase in the stability of tubulin transcripts (3). The processes regulating tubulin transcript accumulation during the cell cycle are unknown.

C. reinhardtii has two genes that encode α-tubulin, *tubA1* and *tubA2*, and two that encode β-tubulin, *tubB1* and *tubB2*. There is a coordinate increase in transcription from all four of these genes following deflagellation. DNA sequence motifs common to all of the tubulin promoters have been postulated to be necessary for increased transcription following deflagellation. These sequence motifs include a GC-rich sequence of 10 to 11 bp located between the TATA box and the transcription initiation site and multiple copies of a short conserved sequence motif that we call the tub box. The 10-bp consensus sequence of the tub box is GCTC(G/C)AAGGC. Multiple sequences that are at least 70% identical to the tub box consensus are found in all of the *tub* genes. In these genes, the tub box motifs are often adjacent to or overlap with one another but occasionally are present as single copies (6). The genes encoding the radial spoke proteins Rsp3, Rsp4, and Rsp6 are also regulated by deflagellation and contain tub box sequence motifs in their promoters (11, 37). Like the *tub* genes, *rsp4* has multiple copies of the tub box motif. However, the promoters of *rsp6* and *rsp3* contain two and one tub box motif, respectively (11). Several other genes also contain tub box sequences. One of the genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcS2*) (17) and another encoding the chaperonin HSP70 (24) have multiple copies of the tub box motif in their promoters. The effect of deflagellation on transcription of these genes is unknown. Furthermore, the *cblp* gene, encoding the β subunit of a G protein, has a single tub box motif in its promoter sequences. Transcription of *cblp* does not increase following deflagellation (31).

We have attempted to identify sequences that regulate transcription of the *tub* genes by examining transcription from mutated copies of the *tubB2* promoter fused to the *ars* reporter gene (12). The *tubB2* gene contains a GC-rich element downstream of the TATA box but preceding the transcription start site and seven copies of the tub box motif immediately upstream of the TATA box (Fig. 1). Previously, we demonstrated that a chimeric gene containing the *tubB2* promoter fused to the arylsulfatase (*ars*) reporter gene was transcribed in *C. reinhardtii* (12) and that temporal changes in the level of the chimeric transcript following deflagellation were similar to that of the endogenous *tubB2* transcripts. In the experiments reported here, we used *tubB2/ars* chimeric genes, with base changes or deletions in the promoter region, to study the importance of *tubB2* *cis*-acting sequences in regulating transcription both after deflagellation and during the cell cycle.

MATERIALS AND METHODS

Cell culture. Strain CC425 (*cw15 arg2*) was obtained from the *Chlamydomonas* Genetics Center at Duke University.

* Corresponding author. Mailing address: Department of Plant Biology, Carnegie Institution of Washington, 290 Panama St., Stanford, CA 94305. Phone: (415) 321-1521. Fax: (415) 321-8725. Electronic mail address: davies@andrew.stanford.edu.

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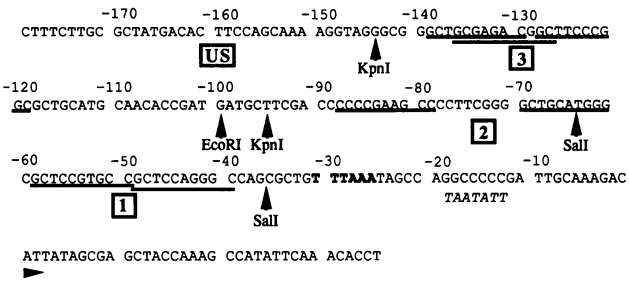


FIG. 1. *tubB2* promoter sequences. The underlined sequences in groups 1, 2, and 3 have at least 70% identity with the 10-bp tub box consensus motif, GCTC(G/C)AAGGC. The positions of introduced restriction endonuclease sites are indicated. *Sal*I sites were introduced at positions −36 and −64, *Kpn*I sites were introduced at positions −95 and −144, and an *Eco*RI site was introduced at −100 (relative to the transcription start site). The GC-rich sequence from position −12 to −18 was changed to an AT-rich sequence (italicized below the original sequence). The transcription start site, at position 1, is indicated by the rightward-pointing arrowhead. The TATA box (from position −25 to −30) is boldfaced.

Asynchronous cultures were grown on Tris-acetate-phosphate (TAP) medium (18) or TAP medium supplemented with 50 μg of arginine per ml. Gametes were made from cultures (5×10^6 to 8×10^6 cells per ml) by washing the cells twice with medium V (36) and resuspending them in medium V for 16 to 20 h. The gametes were deflagellated by pH shock (38). Synchronous cell cultures were maintained on a 12-h/12-h light-dark cycle in TAP medium (35). Cell growth was monitored by counting the cells in a hemocytometer.

Cloning. To examine the function of conserved sequences within the *tubB2* promoter, we generated a series of *tubB2/ars* chimeric genes composed of mutated copies of the *tubB2* promoter fused with the *ars* reporter gene. Mutations in a 1.5-kbp *Eco*RI fragment containing the *tubB2* promoter in pBluescript KS+ were generated by oligonucleotide-directed mutagenesis (2). The oligonucleotide TAATGTCTTTGCA ATAATATTATGGCTATTTAAACAG was used to change the GC-rich region located between the TATA box and the transcription initiation site of the *tubB2* promoter (AGCCAG GCCCCCGATT) to an AT-rich region (AGCCATAATATTA TT) (Fig. 1) (positions −12 to −19). This change ablates the GC-rich region and reduces the spacing between the TATA box and transcription initiation site by 1 nucleotide. The plasmid containing the entire *tubB2* promoter with this mutation was restricted at a *Kpn*I site in the multiple cloning site about 900 bp upstream of the transcription start site and at an *Xho*I site 65 bp downstream of the transcription start site. This fragment (about 1 kbp) was fused to *ars* to generate the new plasmid, ptubB2AT/ars. The oligonucleotides TCTCGCAGC CCGCGGTACCTTTTGCTGG and CGGGGGGTCGAGG TACCATCGGTGTTGCATG were used to introduce *Kpn*I sites, singly, at positions −144 and −95 relative to the *tubB2* transcription initiation site, and the oligonucleotides CG GCACGGAGCGTCGACGCAGCCCCGAAGGG and TGG CTATTTAAACAGTCGACTGGCCCTGGAGC were used to introduce *Sal*I sites at positions −64 and −36, respectively (Fig. 1). To generate the mutant chimeric genes, the mutagenized *tubB2* promoters were cut once at the introduced restriction endonuclease site and once at the *Xho*I site in the 5′-transcribed but untranslated region and fused to the *ars* reporter gene (12). These constructs generated a series of chimeric genes with promoters containing 144, 95, 64, or 36 bp of the *tubB2* sequence upstream of the transcription start site; the plasmids harboring these constructs were designated ptubB2ΔUS/ars, ptubB2ΔUS,3/ars, ptubB2ΔUS,3,2/ars, and ptubB2ΔUS,3,2,1/ars, respectively. Diagrams of these constructs are presented in Fig. 2. The plasmid designations are based on regions deleted from the original *tubB2* promoter. There are three groups of tub box motifs present in the *tubB2* promoter; these are designated 3, 2, and 1 (from 5′ to 3′). The region upstream of these motifs is designated US (upstream sequences). In the plasmid name, any region noted after the Δ symbol has been deleted from the *tubB2/ars* chimeric gene. A construct with the *tubB2* sequence from position −64 to −95

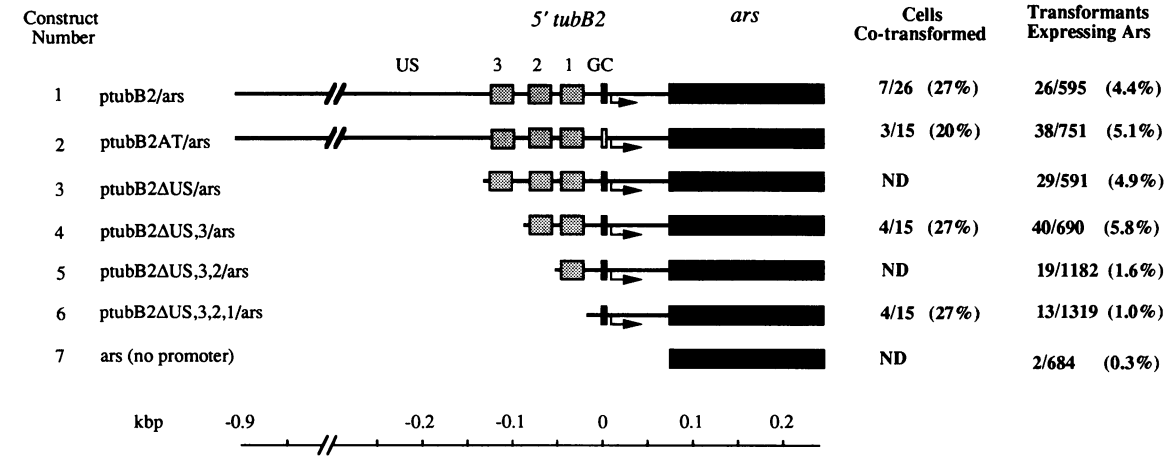


FIG. 2. Schematic drawings of the *tubB2/ars* constructs. 5′ *tubB2* sequences are represented by thin lines, with the shaded boxes representing groups of the tub box motifs. The GC-rich sequence is represented by the small solid box immediately upstream of the transcription start site (arrow). The open box at this position in construct 2 represents an AT-rich sequence. The *ars* sequences are represented by a solid bar. The entire *ars* coding region and 3′ untranslated region are present in all of the constructs, although they are not represented in the schematic drawings. The cotransformation rate, as determined by DNA gel blot analysis, is reported for ptubB2/ars, ptubB2AT/ars, ptubB2ΔUS,3/ars, and ptubB2ΔUS,3,2,1/ars. It is presented as the number of cells cotransformed over the number of transformants examined. ND, not determined. The percentage of total transformants expressing the chimeric gene is reported as the number of cells exhibiting Ars activity on TAP medium over the number of transformants tested.

deleted was generated by fusing a fragment of the *tubB2* promoter from the introduced *Kpn*I site at position −95 to the *Eco*RI site in the multiple cloning site at about −900 with ptubB2ΔUS,3,2/ars. A construct with the *tubB2* sequence from −36 to −64 deleted was generated by fusing a fragment from the introduced *Sal*I site at position −64 to the upstream *Eco*RI site with ptubB2ΔUS,3,2,1/ars. These constructs were designated ptubB2Δ2/ars and ptubB2Δ1/ars, respectively (see Fig. 5A). To generate a *tubB2/ars* chimeric gene with only sequences from position −64 to −100 upstream of the TATA box, we altered the *tubB2* promoter to have a *Sal*I site at position −64 as described above and an *Eco*RI site at position −100 using the oligonucleotide CGGGGGGTCGAGAAT TCATCGGTGTTGCATG. A fragment of this promoter extending from the introduced *Sal*I site at position −64 to the *Kpn*I site at about −900 was ligated into the multiple cloning site upstream of ptubB2ΔUS,3,2,1/ars. The finished construct, ptubB2ΔUS,3,1/ars (see Fig. 5A), was generated by removing sequences upstream of position −100 by cutting at the *Eco*RI sites at positions −100 and −900 and religating the plasmid. Finally, to create a *tubB2/ars* chimeric gene containing only the tub box motifs between positions −64 and −100 in their original position relative to the rest of the promoter, we simultaneously introduced a *Kpn*I site at position −95 (see above) and changed the sequences composing the two tub box motifs between positions −40 and −60 using the oligonucleotide TAAACAGCGCTGGCATATCTTGAATTCT TCGCGCCCATGCAG. This changed the sequence from GCTCCGTGCCGCTCCAGGGC to GCGAAGAATTCAA GATATGC. The plasmid containing the *tubB2/ars* chimeric gene with this promoter, ptubB2X1ΔUS,3/ars (see Fig. 5A), was constructed by cutting the mutagenized *tubB2* promoter once at the *Xho*I site in the transcribed but untranslated region and once at the *Kpn*I site at position −95 and fusing it with the *ars* reporter gene.

The promoter region of each of the chimeric genes was sequenced (29) to confirm the expected modifications.

Transformation. CC425 *mt+* was transformed by the glass bead method (21) with 2 μg of pJD67 (containing the argininosuccinate lyase gene) (12, 14) and an equimolar amount of the *tubB2/ars* constructs. Both plasmids were introduced as supercoiled DNA. After transformation, cells were spread on solid TAP medium (lacking arginine). Transformants grew in the absence of exogenous arginine. To test for the presence of the *tubB2/ars* gene, the transformed colonies were grown on solid TAP medium for 5 to 7 days and sprayed with the chromogenic arylsulfatase (Ars) substrate 5-bromo-4-chloro-3-indolyl sulfate. Colonies expressing Ars had blue halos (12). DNA gel blot hybridization of genomic DNA from the transformants that exhibited Ars activity confirmed the presence of the chimeric gene in the algal genome.

DNA and RNA gel blot analyses. DNA and RNA gel blot analyses were performed as described by Davies et al. (12). The level of hybridization to the blotted RNA was quantitated with a Molecular Dynamics PhosphorImager.

RESULTS

Expression of *tubB2/ars* chimeric genes in asynchronous cultures. To functionally identify sequences involved in controlling expression of the *tubB2* gene, we altered the *tubB2* promoter by changing the GC-rich sequence to an AT-rich region and deleting various segments of the promoter. The GC-rich sequence was changed from GCCAGCCCCCG to GCCATAATATT by oligonucleotide-directed mutagenesis (2) (Fig. 1). The altered promoter was fused to the *ars* reporter gene, and the construct was designated ptubB2AT/ars (Fig. 2).

Upstream of the TATA box of the *tubB2* promoter are multiple copies of the tub box sequence motif. We have arbitrarily assigned these sequences to one of three groups, each containing two or three copies of the tub box motif, and have designated these groups tub box groups 1 to 3. Sequences upstream of the tub boxes have no homology with similar regions in the other *tub* genes; we have designated these upstream sequences US (Fig. 1). US and the tub box groups were deleted from the *tubB2* promoter by digesting at unique restriction endonuclease sites that had been introduced singly at position −144, −95, −64, or −36 relative to the transcription initiation site (Fig. 1) (see Materials and Methods). The mutated promoters were fused to the *ars* reporter gene, forming constructs ptubB2ΔUS/ars, ptubB2ΔUS,3/ars, ptubB2 ΔUS,3,2/ars, and ptubB2ΔUS,3,2,1/ars (Fig. 2).

The chimeric genes were introduced into the cell wall-deficient (*cw15*), arginine auxotrophic (*arg2*) *C. reinhardtii* strain CC425 via cotransformation with the *arg2* gene (encoding argininosuccinate lyase) as the selectable marker (14). Equimolar amounts of plasmid DNA containing the selectable marker and the *tubB2/ars* constructs were used for transformation. To select for transformants, cells were spread on solid TAP medium lacking arginine; transformants were able to grow in the absence of exogenously applied arginine.

To determine the frequency of cotransformation, randomly chosen transformants were examined for the presence of *tubB2/ars* by DNA gel blot hybridization with radiolabeled *ars* DNA. Cell lines containing *ars* hybridizing sequences that were not present in the untransformed strain were considered to be cotransformed. From these analyses, we determined that 20 to 27% of the transformants received at least a portion of the *tubB2/ars* chimeric gene (Fig. 2). No differences in the cotransformation rates were detected when different *tubB2/ars* constructs were used. To determine the percentage of transformants expressing the chimeric gene, we examined randomly chosen transformants for Ars activity. Between 5.8 and 1.0% of the transformants expressed Ars in TAP medium (Fig. 2). Thus, 75 to 90% of the transformants that received the *tubB2/ars* gene did not express Ars activity. A low frequency of transformants expressing Ars is expected because the introduced DNA integrates into the chromosome and the recombination event is likely to occur within the chimeric gene about 70% of the time (since the introduced DNA contains 7 to 8 kbp of *tubB2/ars* sequences and 3 kbp of vector sequences). Recombination within the chimeric gene will probably prevent its expression. In addition, integration of the chimeric gene into the chromosome may result in deletions of portions of the chimeric gene, which will also prevent expression. To determine whether cotransformed cells that were not expressing Ars had rearranged or lost portions of the chimeric gene, we analyzed DNAs from 12 cell lines. DNA gel blot analysis indicated that 10 of the 12 cell lines contained only one copy of the *tubB2/ars* gene; the other 2 cell lines received two copies. To determine whether the *tubB2/ars* chimeric gene was intact in these cell lines, DNA was digested with enzymes that cut either once in the 5′ portion of the gene and once at an internal position or once in the 3′ portion of the chimeric gene and once internally. Digested genomic DNA was fractionated by electrophoresis, blotted onto nitrocellulose, and hybridized with sequences from either the 5′ or the 3′ portion of *ars*. In genomic DNA containing an intact copy of the chimeric gene, DNA fragments hybridizing with the 5′ and 3′ portions of *ars* will be the same sizes as the corresponding fragments from the *tubB2/ars* plasmid. However, genomic DNA containing a dis-

rupted copy of the chimeric gene will contain fragments that are either larger or smaller than fragments from the plasmid DNA. In all 12 cell lines analyzed in this manner, DNA fragments from the 5′ or 3′ end of the chimeric gene were either larger or smaller than the predicted size. This indicated that a portion of the chimeric gene was lost or rearranged during its integration into the chromosome (data not shown) and would account for the finding that these cell lines do not express the chimeric gene.

The number of transformants expressing Ars was dependent on the construct introduced. Cotransformation with ptubB2/ars, ptubB2AT/ars, ptubB2ΔUS/ars, and ptubB2ΔUS,3/ars resulted in a higher percentage of Ars-expressing transformants than did cotransformation with ptubB2ΔUS,3,2/ars and ptubB2ΔUS,3,2,1/ars (Fig. 2) (5.8 to 4.4% compared with 1.6 and 1.0%, respectively). Because ptubB2ΔUS,3,2/ars and ptubB2ΔUS,3,2,1/ars were apparently cotransformed at the same frequency as the other constructs, it may be that expression from ptubB2ΔUS,3,2/ars and ptubB2ΔUS,3,2,1/ars was more sensitive to the site of integration into the chromosome than expression from the other constructs.

As a control, we introduced the *ars* reporter gene without a promoter and analyzed over 600 transformants for Ars expression. We found only two that exhibited Ars activity in TAP medium; the levels of Ars activity in these two strains were much lower than that observed for transformants in which the chimeric gene contained a promoter. Ars expression in these cells probably resulted from integration of the *ars* gene into the chromosome near sequences that served as a promoter or from the generation of a mutation causing Ars to be constitutively expressed. When no *ars* gene was introduced, only 1 in 5,000 transformants exhibited Ars activity (13).

DNA gel blot analyses of all strains tested confirmed that transformants expressing Ars activity in TAP medium after cotransformation with a *tubB2/ars* construct contained at least one copy of the chimeric gene. Specifically, 31 cell lines were examined, and 21 had only one copy of the *tubB2/ars* chimeric gene while 7 had two copies and 3 had more than two copies. Some of the Ars-expressing cell lines were further characterized to determine whether they received the entire 5′ portion of the chimeric gene. Most (13 of 15) of the cell lines expressing Ars after transformation with constructs containing 900 bp upstream of the transcription start site (ptubB2/ars, ptubB2AT/ars, ptubB2Δ2/ars, and ptubB2Δ1/ars) contained at least 800 bp of the upstream sequences, and the others contained at least 300 bp. Most (11 of 12) of the cells expressing Ars after transformation with chimeric genes having 144 bp or fewer of *tubB2* promoter sequences (ptubB2ΔUS/ars, ptubB2ΔUS,3/ars, ptubB2ΔUS,3,2/ars, and ptubB2ΔUS,3,2,1/ars) contained the entire portion of the *tubB2* promoter present in the original chimeric gene (data not shown).

RNA gel blot analyses of Ars-expressing strains confirmed that the chimeric constructs were transcribed. The untransformed strain, CC425, accumulated no *ars* mRNA in vegetative cells growing in TAP medium (12) (Fig. 3A, lane 1), while transformants expressing Ars activity accumulated transcripts that hybridized with the *ars* cDNA (Fig. 3A, lanes 2 to 7). Hybridization with *tubB2*-specific sequences detected both the endogenous *tubB2* transcript and the chimeric transcript (Fig. 3B). Because there are only 65 bases of the *tubB2* sequence in the chimeric transcript, hybridization with *tubB2* was much weaker than with *ars*. However, long exposures of the blot hybridized with *tubB2* DNA confirmed the presence of the chimeric transcript (data not shown).

To determine whether the mutations in the *tubB2* promoter affect transcription during vegetative growth, we compared

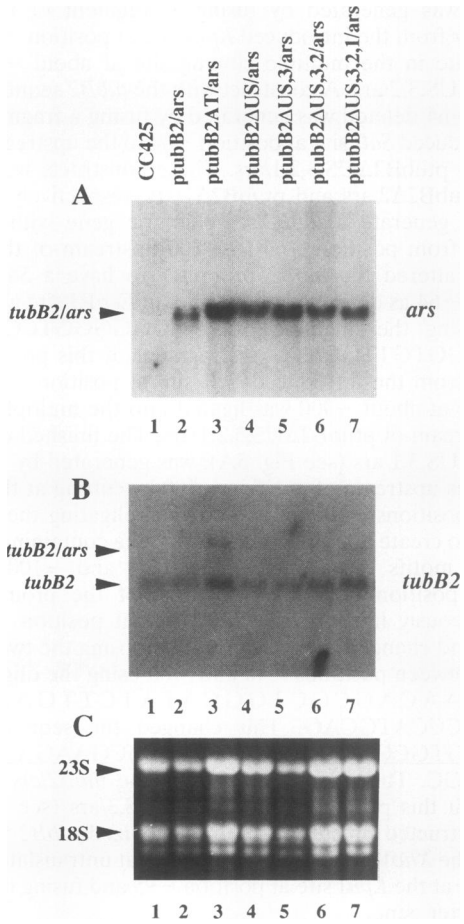
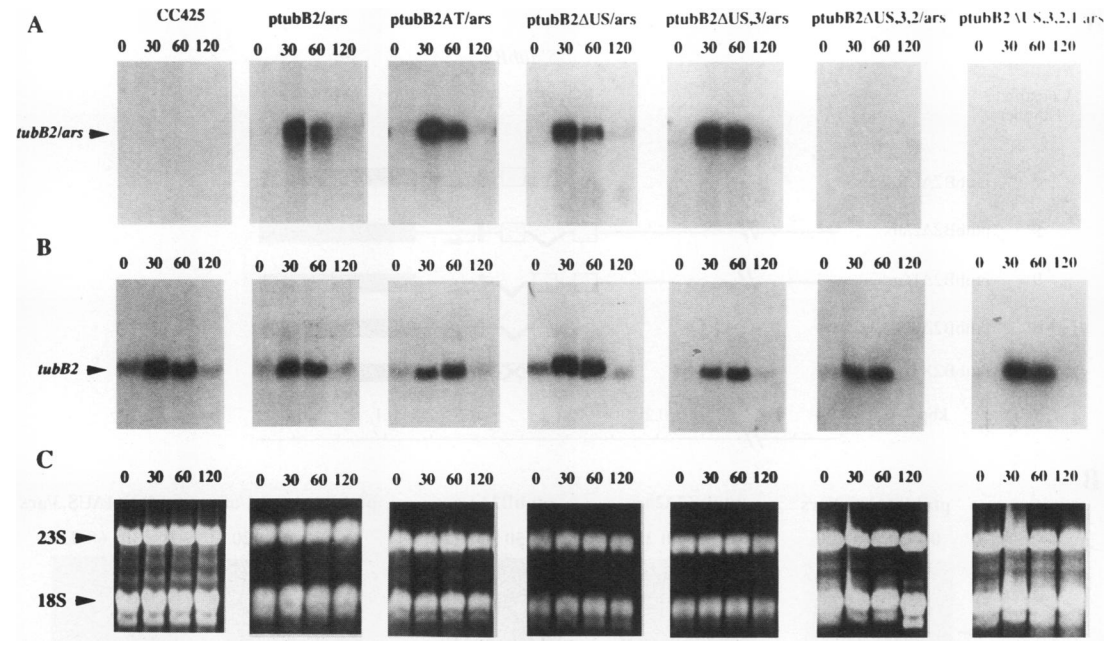


FIG. 3. RNA gel blot analyses of transformants during vegetative growth. Total RNA from cells expressing each construct (Fig. 2) was isolated, separated in agarose gels by electrophoresis, transferred onto nitrocellulose filters, and hybridized with the *ars* cDNA (A) or sequences that are specific for the first 65 bases of the 5′-transcribed but untranslated region of the *tubB2* gene (B). (C) Ethidium-stained gel containing approximately 10 μg of RNA in each lane.

chimeric transcript accumulation levels in several transformants expressing each construct. Since transformants containing multiple copies of the chimeric gene may also be expressing more than one gene, we compared transcript levels only among transformants containing a single copy of *tubB2/ars*. The difference in transcript accumulation within transformants expressing the same construct was as great as the difference among transformants expressing different constructs. Thus, within the limits of our assay, we could detect no *cis*-acting element between bp −900 and −36 (relative to the transcription initiation site) that greatly enhances or represses transcription of *tubB2* during asynchronous, vegetative growth. Since the level of the chimeric mRNA that accumulated in these constructs is approximately 10% of that observed for the endogenous *tubB2* transcript, it is possible that we eliminated an enhancer element during construction of ptubB2/ars (the enhancer element could be more than 900 bp upstream or within the coding or 3′ untranslated region of the *tubB2* gene).

Identification of sequences necessary for induced transcription following deflagellation. To test whether the GC-rich sequences and tub box motifs are necessary for increased transcription following deflagellation, we performed RNA gel



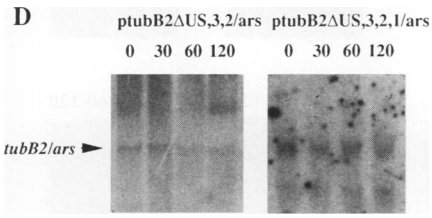


FIG. 4. RNA gel blot analyses of transformants expressing chimeric genes (designations are as in Fig. 2) after deflagellation. Gametic cells were deflagellated by pH shock, and RNA was isolated 0, 30, 60, and 120 min after deflagellation. After separation by electrophoresis, the RNA was transferred to nitrocellulose filters and hybridized with *ars* cDNA (A) and *tubB2*-specific sequences (B). The RNA blots in panels A and B were exposed to film for 16 to 20 h. (C) Ethidium-stained gel, with each lane containing approximately 5 μg of RNA. The RNA blots in panel D were hybridized with the *ars* cDNA and exposed to film for 3 to 5 days. The more slowly migrating bands present in ptubB2ΔUS,3,2/ars have not been identified. Numbers of transformants examined: ptubB2/ars, 9; ptubB2AT/ars, 5; ptubB2ΔUS/ars, 11; ptubB2ΔUS,3/ars, 5; ptubB2ΔUS,3,2/ars, 8; ptubB2ΔUS,3,2,1/ars, 3. RNA accumulation levels in all transformants receiving a complete copy of the same chimeric gene (as determined by DNA gel blot analysis) were similar.

blot analyses on RNA isolated from transformants at various times following deflagellation. Since gametic cells were able to express the chimeric genes and exhibited a greater difference in the levels of both *tubB2* and *tubB2/ars* mRNAs accumulated before and after deflagellation than vegetative cells (data not shown), deflagellation experiments were performed on gametes. At least three independent transformants expressing the chimeric genes were deflagellated and examined for accumulation of *tubB2/ars* mRNA. Representative autoradiograms are presented in Fig. 4. As previously demonstrated (12), transcripts from ptubB2/ars (previously called pJD55) and the endogenous *tubB2* gene accumulate similarly in response to deflagellation. The accumulation of *tubB2* transcripts is initially low and transiently increases to a peak between 30 and 60 min following deflagellation. In transformants expressing ptubB2AT/ars, in which the GC-rich sequence was changed to an AT-rich sequence, *tubB2/ars* mRNA also accumulated with kinetics similar to those of the *tubB2* mRNA. Furthermore, removing sequences upstream of the tub box motifs had little effect on the kinetics or level of transcript accumulation following deflagellation (ptubB2ΔUS/ars [Fig. 4A]). Comparing the abundance of *tubB2/ars* mRNA in transformants expressing constructs with three (ptubB2ΔUS/ars), two (ptubB2ΔUS,3/ars), or one (ptubB2ΔUS,3,2/ars) of the tub box groups or without any tub box motifs (ptubB2ΔUS,3,2,1/ars) indicated that sequences constituting tub box groups 1 and 2 were sufficient to allow transcript accumulation in response to deflagellation (ptubB2ΔUS,3/ars). Constructs with only tub box group 1 (ptubB2ΔUS,3,2/ars) or no tub box motifs (ptubB2ΔUS,3,2,1/ars) did not show elevated transcript accumulation following deflagellation (Fig. 4A). All transformants receiving the complete chimeric gene (as determined by DNA gel blot analysis) responded similarly. Autoradiograms exposed for a longer time than those displayed in Fig. 4A confirmed that low-level, constitutive expression of the chimeric gene occurred in cells harboring ptubB2ΔUS,3,2/ars and ptubB2ΔUS,3,2,1/ars (Fig. 4D). Since group 1 contains only two tub box motifs and groups 1 and 2 together contain four, there may be a minimum number of these motifs required for enhanced transcription following deflagellation.

To examine the variability in transcript accumulation in transformants that exhibit elevated expression of the chimeric gene upon deflagellation, we compared accumulation levels of the chimeric transcripts 30 min following deflagellation. At least three individuals with the same chimeric gene were tested. The variation in accumulation of chimeric transcript accumulated in transformants expressing the same gene was as great as the variation among transformants expressing different genes (about threefold) (data not shown). The same result was obtained if measurements were confined to transformants containing a single copy of the chimeric gene.

The data presented above demonstrate that we have identi-

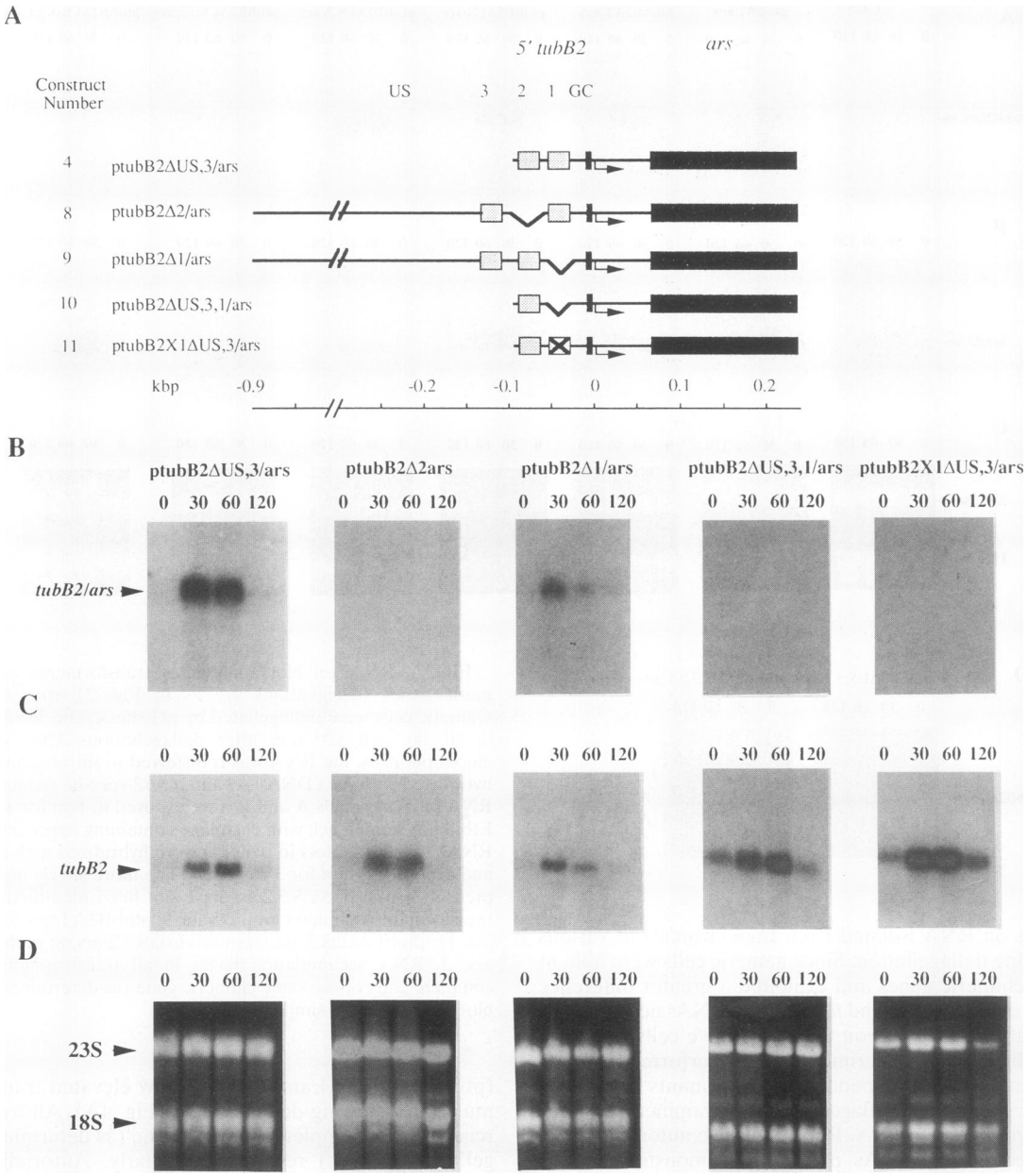


FIG. 5. RNA gel blot analyses of transformants expressing chimeric genes after deflagellation. (A) Schematic drawings of the *tubB2/ars* constructs as in Fig. 2. Gametic cells were deflagellated by pH shock, and RNA was isolated 0, 30, 60, and 120 min after deflagellation. After separation by electrophoresis, the RNA was transferred to nitrocellulose filters and hybridized with *ars* cDNA (B) and *tubB2*-specific sequences (C). (D) Ethidium-stained gel, with each lane containing approximately 5 μg of RNA. The data shown for ptubB2ΔUS,3/ars are the same as those in Fig. 4. Numbers of transformants examined: ptubB2ΔUS,3/ars, ptubB2Δ2/ars, ptubB2Δ1/ars, and ptubB2ΔUS,3,1/ars, five each; ptubB2X1ΔUS,3/ars, four. RNA accumulation levels in all transformants expressing the same construct were similar.

fied *cis*-acting elements that are important for elevated transcription following deflagellation. However, we have not identified elements that affect the level of this induction.

Multiple copies of the tub box motif are necessary for *tubB2/ars* mRNA accumulation following deflagellation. Since there are three tub box groups in the *tubB2* promoter and the sequences within tub box groups 1 and 2 are sufficient for induced transcription following deflagellation (ptubB2ΔUS,3/ars [Fig. 4 and 5]), we tested if combinations of other tub box groups were also sufficient to induce transcription. Chimeric genes with tub box groups 1 and 3 but lacking group 2 (ptubB2Δ2/ars) (Fig. 5A) or with groups 2 and 3 but lacking group 1 (ptubB2Δ1/ars) (Fig. 5A) were introduced into *C. reinhardtii*. Cells expressing the chimeric genes were identified by assaying for Ars activity in sulfur-sufficient medium, and expression was confirmed by RNA gel blot analysis. Cell lines expressing Ars were analyzed for induced accumulation of the *tubB2/ars* mRNA following deflagellation (Fig. 5B). No elevated accumulation of the chimeric transcript occurred upon deflagellation if the *tubB2* promoter on the introduced con-

struct contained groups 1 and 3 (ptubB2Δ2/ars). However, when groups 2 and 3 were present (ptubB2Δ1/ars), transcription from the chimeric gene increased upon deflagellation.

Since tub box group 2 was present in all of the constructs that responded to deflagellation, we tested whether it alone was sufficient for deflagellation-induced transcription. A *tubB2/ars* chimeric gene in which only tub box group 2 was upstream of the TATA box (ptubB2ΔUS,3,1/ars) (Fig. 5A) was introduced into *C. reinhardtii*. Transformants expressing this gene in vegetative cells were tested for induced transcription following deflagellation. None of these cell lines exhibited elevated transcription of the chimeric gene following deflagellation (ptubB2ΔUS,3,1/ars [Fig. 5B]). As a final test to determine if tub box group 2 was sufficient for induced transcription following deflagellation, the construct ptubB2X1ΔUS,3/ars was generated and tested. This construct contains no *tubB2* sequences upstream of group 2, and the sequences of group 1 were changed from GCTCCGTGCCGCTCCAGGGC to GC GAAGAATTCAACATATGC. This change ablated the sequence of tub box group 1 but maintained the same number of bases between group 2 and the TATA box. This chimeric gene is identical to ptubB2ΔUS,3/ars, which is induced following deflagellation (ptubB2ΔUS,3/ars [Fig. 5]), except for the altered tub box group 1. None of the transformants that showed constitutive expression of the chimeric gene (ptubB2X1ΔUS,3/ars) exhibited induced transcription following deflagellation. Thus, tub box group 2 alone is not sufficient for induced transcription following deflagellation. However, a combination of tub box groups 2 and 1, as in ptubB2ΔUS,3/ars, or groups 2 and 3, as in ptubB2Δ1/ars, is sufficient for deflagellation-induced transcription.

***tubB2* promoter sequences necessary for regulated expression during the cell cycle.** Since both tubulin synthesis and transcript accumulation (for both α-tubulin and β-tubulin) are modulated during the cell cycle (1, 35), we examined whether accumulation of transcripts from the chimeric gene and that from the endogenous *tubB2* gene were regulated similarly during the cell cycle. Cell growth was synchronized by a 12-h light/12-h dark cycle for several days (at least 4), and RNA was isolated 2, 6, 10, 14, 18 and 22 h after the start of the light period. The synchrony of the cells is depicted in Fig. 6A, which shows an increase in cell density that begins at the start of the dark period. RNA gel blot analyses using a *tubB2*-specific DNA as a probe demonstrated that the *tubB2* mRNA accumulated maximally at 18 h (Fig. 6C), when most of the cells were dividing. Accumulation of the chimeric transcript from ptubB2/ars was temporally different; its accumulation was cell cycle dependent but was maximal earlier in the cycle (6 to 14 h). Transformants containing mutations of the *tubB2* promoter were also examined to determine which promoter sequences were important for maintaining the cell cycle-regulated accumulation of the *tubB2/ars* mRNA. Transformants expressing constructs lacking US and tub box group 3 (ptubB2ΔUS,3/ars) accumulated *tubB2/ars* mRNA with a timing similar to the timing for those that harbored the entire promoter. The transformant expressing the chimeric gene lacking US and tub box groups 2 and 3 (ptubB2ΔUS,3,2/ars) accumulated the *tubB2/ars* transcript with some cell cycle dependence, but the level of the transcript did not vary as much throughout the cell cycle as that of the construct with the full *tubB2* promoter (900-bp fragment). Finally, transformants expressing the ptubB2ΔUS,3,2,1/ars construct accumulated the *tubB2/ars* transcript with much less cell cycle dependence (Fig. 6B).

DISCUSSION

We have measured transcript accumulation from constructs with various modified *tubB2* promoters fused to the *ars* reporter gene in transformed *C. reinhardtii*. To our knowledge, this is the first detailed promoter analysis using this organism. Accumulation of the *tubB2* transcript, as well as transcripts of other tubulin-encoding genes in this organism, increases following deflagellation, during which time there is rapid flagellar regeneration. The α- and β-tubulin genes of *C. reinhardtii* have similar promoter sequences; there is a GC-rich element between the TATA box and the transcription initiation site and multiple copies of a 10-bp conserved sequence (the tub box motif) (6). We have examined the effect of changing the GC-rich sequence or deleting the tub box sequences on the transcription of chimeric genes in asynchronous and synchronous vegetative cells as well as in deflagellated gametic cells.

We have reached several conclusions based on the work presented here. Altering the GC-rich sequence downstream of the TATA box in the *tubB2* promoter to an AT-rich sequence does not greatly affect the expression of *tubB2/ars* in an asynchronous cell culture. A similar GC-rich sequence is present in a number of the genes of *C. reinhardtii*, including genes encoding the other tubulin subunits (6), the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (17), a 70-kDa heat shock protein (24), a radial spoke protein (Rsp3) (37), a chlorophyll *a/b*-binding protein (19), and a protein with homology to the β subunit of a G protein (31). The presence of this sequence in so many genes led investigators to speculate that the GC-rich region was important for transcription. It was previously reported by Bandziulis and Rosenbaum (4) that interrupting the GC-rich sequence in the *tubA1* promoter with a 12-bp segment of DNA severely inhibited transcription of a *tubA1/cat* chimeric gene in *Xenopus* oocytes. Our results suggest that the GC-rich region is not important for constitutive transcription of the *tubB2* gene in vegetative cultures. There are a number of differences between our studies and those of Bandziulis and Rosenbaum (4). We analyzed expression from the *tubB2* promoter fused to the *ars* reporter gene in *C. reinhardtii*, while Bandziulis and Rosenbaum (4) worked with the *tubA1* promoter fused to the *cat* reporter gene and examined expression in *Xenopus* oocytes. Perhaps more importantly, we changed the GC-rich sequences by oligonucleotide-directed mutagenesis and reduced the number of bases between the TATA box and the transcription initiation site by only one. Bandziulis and Rosenbaum (4) introduced a 12-bp segment of DNA into the middle of the GC-rich sequence; this substantial change in the distance between the TATA box and the transcription start site may significantly affect transcription (25).

Our results also indicate that in asynchronous, vegetative cultures the *tubB2* promoter containing only 35 bp upstream of the transcription initiation site is sufficient to drive constitutive transcription of the chimeric gene with an efficiency similar to that of the promoter containing 900 bp. Since all tub box motifs are upstream of −35, these sequences do not appear to serve as enhancers or repressors of transcription during vegetative growth.

In addition to examining expression of the *tubB2* gene in vegetative cultures, we explored the role of the GC-rich region and the tub boxes on gene expression following deflagellation of gametic cells. When the GC-rich sequences were converted to sequences enriched for A and T, there was no great effect on the induced transcript accumulation of the *tubB2/ars* chimeric gene following deflagellation. Chimeric genes containing deletions of tub box sequences were also tested for their ability to

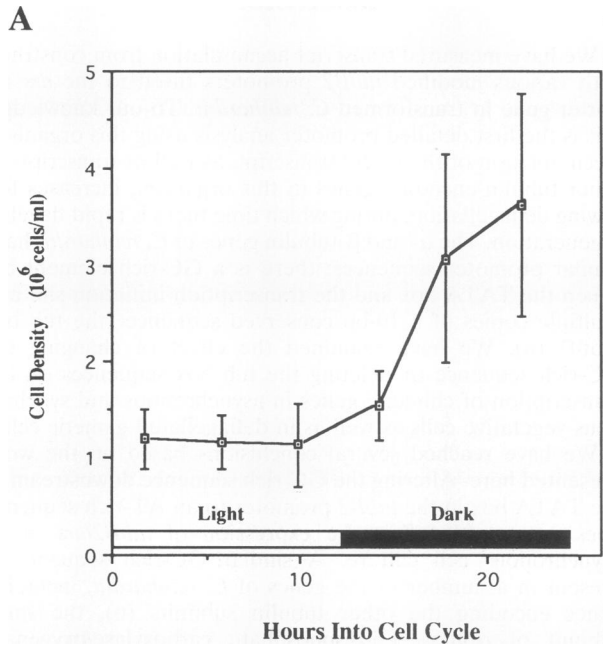
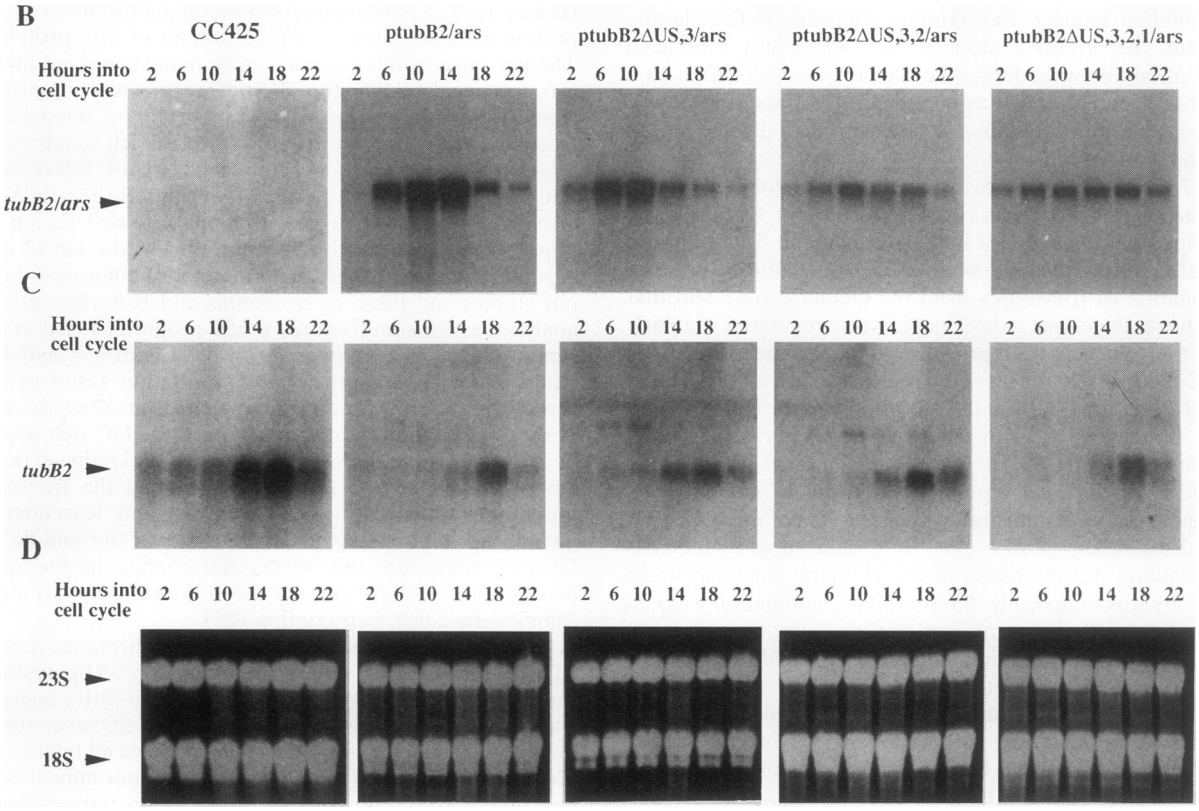


FIG. 6. RNA gel blot analysis of transformants expressing chimeric genes (designations are as in Fig. 2) during synchronous cell growth. Cells were grown in TAP medium on a 12-h light/12-h dark cycle. (A) Average cell density during the cell cycle of five cultures that had starting densities of approximately 10^6 cells per ml. The cell densities were determined by counting the cells in a hemocytometer. Bars, standard errors of the means. Light and dark periods are indicated. RNA was isolated 2, 6, 10, 14, 18, and 22 h from the start of the light period. RNA was separated by electrophoresis in agarose gels, transferred onto nitrocellulose filters, and hybridized with *ars* cDNA (B) or *tubB2*-specific sequences (C). (D) Ethidium-stained gels with each lane containing approximately 10 μg of RNA. Numbers of transformants examined: ptubB2/ars, four; ptubB2ΔUS,3/ars, two; ptubB2ΔUS,3,2/ars, one; ptubB2ΔUS,3,2,1/ars, three. RNA accumulation levels in all transformants expressing the same construct were similar.



induce transcript accumulation following deflagellation. The inclusion of two or three groups of tub box sequences in the constructs was sufficient for induction following deflagellation; however, no elevated transcript accumulation was observed if the introduced construct contained fewer than two groups of tub box motifs. Transcript accumulation levels in cells harboring constructs containing the first and second tub box groups, the second and third groups plus 750 bp of US, all three groups, and all three groups plus 750 bp of US were similar. Since all the chimeric genes that were responsive to deflagellation contained tub box group 2, we tested whether it alone was sufficient to cause elevated transcription following deflagellation. Neither a construct containing only tub box group 2 fused directly upstream of the TATA box (ptubB2ΔUS,3,1/ars)

nor a construct in which tub box group 2 was maintained at its original position in the *tubB2* promoter but with the sequences of tub box group 1 changed (ptubB2X1ΔUS,3/ars) could induce elevated transcription following deflagellation. Because two constructs with different sets of tub boxes (ptubB2ΔUS,3/ars and ptubB2Δ1/ars, containing four and five tub box motifs, respectively) showed increased transcript accumulation following deflagellation while three different constructs with only one tub box group (ptubB2ΔUS,3,2/ars, ptubB2ΔUS,3,1/ars, and ptubB2X1ΔUS,3/ars, containing two tub box sequence motifs) did not, we speculate that a minimal number of tub box sequences are required for increased transcription from tubulin gene promoters following deflagellation. The construct in which only tub box group 2 was deleted (ptubB2Δ2/ars) has five tub box sequence motifs but could not support induced transcription. In this construct the adjacent tub box motifs (from groups 1 and 3) are farther apart than in the other constructs and may be on separate sides of the DNA helix. Therefore, we suggest that the distance between adjacent tub box motifs, and perhaps their orientation with respect to each other, is critical for induced transcription.

All *tub* genes have multiple copies of tub boxes, and their transcription may be regulated by a mechanism similar to the one regulating transcription of *tubB2*. However, the *rsp3* and *rsp6* genes, whose transcripts increase following deflagellation, have only one and two copies of the tub box sequence motif, respectively. Our data suggest that these sequences are not sufficient to support induced transcription following deflagellation. Thus, other sequence motifs may be involved in regulating the accumulation of transcripts from these genes.

Accumulation of both the α- and the β-tubulin transcripts is observed following deflagellation of *C. reinhardtii* (7, 23, 32) and after deciliation of *Tetrahymena pyriformis* (33). During this time large quantities of tubulin are required for the regeneration of flagella or cilia. The correlation of the accumulation of tubulin mRNA and the amount of tubulin required by the cell raises the possibility of autoregulatory control of tubulin synthesis. In animal cells the stability of β-tubulin transcripts is maintained by an autoregulatory system in which the level of unpolymerized tubulin subunits within the cell alters the stability of β-tubulin mRNA (10). When the level of unpolymerized tubulin subunits is high, the β-tubulin transcript is unstable. Conversely, when the concentration of unpolymerized tubulin is low, the transcript is stable. Thus, according to the model, when tubulin is rapidly polymerizing into microtubules or flagella, the concentration of unpolymerized tubulin within the cell falls and β-tubulin mRNA is stabilized. Once these structures are completed, the concentration of unpolymerized tubulin within the cell increases and the β-tubulin transcripts become unstable. In *C. reinhardtii*, it appears that *tubB2* mRNA accumulation following deflagellation is controlled primarily by transcription, since deleting sequences in the promoter prevents the increased accumulation of transcripts. Furthermore, it is unlikely that the level of unpolymerized tubulin mediates the transcriptional regulation of *tubB2* following deflagellation, since induced accumulation of *tubB2* mRNA continues even when flagellar growth is inhibited by adding colchicine or withholding Ca^{2+} ions (9). Under these conditions the concentration of unpolymerized tubulin within the cell should be high.

The accumulation of transcripts from both the α- and the β-tubulin genes in *C. reinhardtii* is also regulated over the course of the cell cycle (1). This regulation may be needed to coordinate tubulin synthesis with the formation of the mitotic spindle apparatus and the assembly of new flagella. Cell cycle-regulated expression of tubulin has not been observed in many organisms. *Physarum polycephalum* increases accumulation of the α- and β-tubulin transcripts about 40-fold prior to mitosis (8, 30), while *Tetrahymena thermophila* (22) and HeLa cells (5) increase tubulin mRNA accumulation only 2-fold. In *Aspergillus nidulans*, there is no change in the level of *tub* mRNA during cell division (15).

Our results demonstrate that *tubB2/ars* transcripts accumulate and decline earlier in the cycle than the *tubB2* mRNA. It is unclear what is causing this timing difference, although there are a number of possible explanations. Transcription of the chimeric gene during the cell cycle may be identical to that of the endogenous *tubB2* gene, but the stability of the endogenous *tubB2* transcript may be low early in the cycle (e.g., 2 and 6 h) and increase at later times (e.g., 14 and 18 h). As mentioned previously, the stability of β-tubulin transcripts in mammalian cells is controlled by the pool of unpolymerized tubulin within the cell (10), an effect mediated by the presence of the first four amino acids at the N terminus of the β-tubulin polypeptide (10, 16, 39). The *tubB2* gene of *C. reinhardtii* encodes the same four N-terminal amino acids, suggesting that there may be some control of *tubB2* mRNA stability by the pool of unpolymerized tubulin subunits under certain conditions. These four amino acids are not encoded by the transcript of the chimeric gene, and if cell cycle control of *tubB2* mRNA accumulation involves this sequence, the stability of the chimeric transcript will be different from that of the endogenous *tubB2* transcript. The net effect may be to shift the time of maximal transcript accumulation. An alternative possibility is that some sequences involved in controlling the timing of transcription of the *tubB2* gene were lost during construction of the *tubB2/ars* chimeric gene. Such a result was obtained with sequences controlling the cell cycle-regulated transcription of the *Saccharomyces cerevisiae* histone H2A and H2B genes. These genes are regulated by both positive- and negative-control elements. When only the negative-control element was placed upstream of a constitutively transcribed reporter gene, transcripts accumulated in a cell cycle-regulated manner. However, maximal accumulation of the reporter transcript occurred earlier in the cell cycle than the accumulation of the endogenous H2B transcript (27).

In *C. reinhardtii*, both during cell division and following deflagellation, new flagella are synthesized with the concomitant increase in the expression of the *tub* genes. Our results suggest that deleting tub box sequences alters the regulated expression of *tubB2* both during cell division and following deflagellation. During the cell cycle the *tubB2/ars* chimeric genes containing the upstream sequences and all seven of the tub box motifs (ptubB2/ars) or only four tub box motifs (ptubB2ΔUS,3/ars) are regulated in a cell cycle-dependent manner, but chimeric genes with two (ptubB2ΔUS,3,2/ars) or no (ptubB2ΔUS,3,2,1/ars) tub box sequences show little variation in expression. Since following deflagellation ptubB2/ars is regulated similarly to the endogenous *tubB2* but its timing of expression during the cell cycle is different, at least some of the signals responsible for elevated *tubB2* mRNA accumulation following deflagellation are different from those that stimulate *tubB2* mRNA accumulation during the cell cycle.

Because multiple copies of the tub box sequence motif appear to be required for induced transcription following deflagellation, it is likely that some multimeric form of a transcription factor may bind to these sequences to enhance transcription. The significance of the overlapping tub boxes is not clear, although they may facilitate the binding of transcription factors and may optimize protein-protein interactions that are required for enhanced transcription.

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