α-Tubulin Minichromosome Promoters in the Stichotrichous Ciliate Stylonychia lemnae

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Ciliated protists are model organisms for a number of molecular phenomena including telomerase function, self-splicing introns, and an RNA interference-related mechanism in programmed DNA elimination. Despite this relevance, our knowledge about promoters and transcriptional regulation in these organisms is very limited. The macronuclear genome of stichotrichous ciliates consists of minichromosomes which typically encode a single gene. The 5′ nontranscribed spacers are usually no longer than 400 bp and highly suitable for promoter characterization. We used microinjection of two artificial and differently tagged α1 tubulin minichromosomes into the macronucleus of Stylonychia lemnae as a means to characterize in detail the corresponding promoter. Clonal cell lines that stably maintained both minichromosomes were generated, enabling comparative expression analysis by primer extension assays. Deletion and block substitution mutations of one of the minichromosomes revealed a TATA-like element, a putative initiator element, and two distinct upstream sequence elements (USEs). Determination of transcription initiation sites and a sequence alignment indicated that both TATA-like and initiator elements are conserved components of S. lemnae minichromosomes, whereas the USEs appear to be specific for the α1 tubulin minichromosome. The α2 tubulin minichromosome promoter is very short, comprising the two proximal elements but not the USEs. Despite the latter finding, up-regulation of α-tubulin expression in cells treated with concanavalin A activated the α2 but not the α1 tubulin promoter. These results therefore show that gene expression regulation in S. lemnae occurs at the level of transcription initiation on the basis of structurally different promoters.

In the past, research on ciliates has revealed generally important discoveries such as self-splicing introns (18), telomerase (9), and, recently, small RNAs involved in DNA elimination (26). Despite this relevance and despite evidence that ciliates regulate their genes at the transcriptional level (2, 36, 37), determinants of transcription in these organisms have not been a major focus. In Tetrahymena thermophila, promoter elements have been experimentally identified in the rRNA gene (6, 25), the telomerase RNA gene (11), and the RAD51 gene (35). However, no detailed analysis of a ciliate promoter has been achieved thus far. In ciliates, gene expression is restricted to macronuclei, which develop from micronuclei after conjugation. In this process, micronuclear DNA is fragmented, in part eliminated, and macronucleus-destined DNA is amplified (reviewed in reference 15). In stichotrichous ciliates, the extent of these DNA manipulations is extreme. For example, in stichotrichous ciliates, the extent of these DNA manipulations is extreme. For example, in stichotrichous ciliates, the extent of these DNA manipulations is extreme. For example, in stichotrichous ciliates, the extent of these DNA manipulations is extreme. For example, in stichotrichous ciliates, the extent of these DNA manipulations is extreme. For example, in stichotrichous ciliates, the extent of these DNA manipulations is extreme. For example, in stichotrichous ciliates, the extent of these DNA manipulations is extreme. For example, in stichotrichous ciliates, the extent of these DNA manipulations is extreme. For example, in stichotrichous ciliates, the extent of these DNA manipulations is extreme. For example, in stichotrichous ciliates, the extent of these DNA manipulations is extreme. For example, in stichotrichous ciliates, the extent of these DNA manipulations is extreme. For example, in stichotrichous ciliates, the extent of these DNA manipulations is extreme. For example, in stichotrichous ciliates, the extent of these DNA manipulations is extreme. For example, in stichotrichous ciliates, the extent of these DNA manipulations is extreme. For example, in stichotrichous ciliates, the extent of these DNA manipulations is extreme. For example, in stichotrichous ciliates, the extent of these DNA manipulations is extreme. For example, in stichotrichous ciliates, the extent of these DNA manipulations is extreme. For example, in stichotrichous ciliates, the extent of these DNA manipulations is extreme.

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Here we provide a functional in vivo analysis of the S. lemnae α1 and α2 tubulin minichromosome promoters. Clonal cell lines were generated by microinjecting an equimolar mix of two artificial and differently tagged α-tubulin minichromosomes into the macronuclei of individual cells. While the promoter region of one minichromosome was systematically mutated, the promoter of the other minichromosome was left intact. A direct comparison of the expression levels from the two minichromosomes revealed a promoter structure for the α1 tubulin minichromosome that comprised a putative initiator element, a TATA-like element, and two upstream sequence elements (USEs) involved in transcription activation. The short α2 tubulin promoter does not contain the USEs but nevertheless was found to harbor a determinant for up-regulating α-tubulin expression upon concanavalin A treatment.

MATERIALS AND METHODS

Strains and culturing of S. lemnae. Experiments were conducted either with S. lemnae strain S1, which was obtained by crossing two strains originally isolated at Peterhof near St. Petersburg, Russia, or with strain 27, which was acquired by crossing strains isolated at Dorum, Germany. Cells were cultured as described previously (1).
DNA construction. The bases for Tuba1-st (short-tag) minichromosome generation and mutation were constructed pTuba1-g-st (33) and pTuba1-lt. These pTZ18U vectors contained, inserted into their Smal restriction sites, the complete double-stranded S. lemnæ α1 tubulin minichromosome sequence fused to an Apal restriction site on either end. Besides several inserted restriction sites (33), the main manipulation of the minichromosomal sequence was the insertion of the 19-nucleotide tag sequence 5'-TTCATGATGGCCGACAG-3' (it includes an NcoI restriction site) at position +103 relative to the transcription initiation site (TIS). pTuba1-st is a derivative of pTuba1-g-st and contains an additional Smal restriction site immediately after the 5′ telomeric repeats. For deletions del1, del2, and del3, the sequence between the pTuba1-st Smal and NcoI sites was replaced by corresponding PCR products. del4 was generated by excision of the sequence between the Smal and an endogenous SpeI site. For block substitutions, mutations were generated by overlapping PCR and inserted into the same restriction sites of pTuba1-st. In the same way, the 5′ nontranscribed region of the α1 tubulin minichromosome was replaced by its α2 tubulin counterpart in constructing pTuba1-st-p2. Plasmid clones containing α2, β1, β2, cathepsin L, and heat shock protein 70 (Hsp70) minichromosomes were kindly provided by Günter Steinbrück and are described elsewhere (5, 13; GenBank accession numbers for the Hsp70 and cathepsin L minichromosomes are AF227962 and AF227971, respectively).

Transfection of S. lemnæ. Linear Tub1-1t (long-tag) and Tuba1-st minichromosomes were obtained by restriction digestion of the corresponding plasmids and Klenow treatment as described previously (33). They possessed the correct double-stranded telomeric repeats on either end but no single-stranded overhangs. It and st minichromosomes were mixed in equimolar amounts and microinjected directly into the macronuclei of immobilized S. lemnæ cells as described elsewhere (32, 33). For each mutation, a minimum of three clonal cell lines were raised from individual transfected cells.

Concanavalin A treatment. Concanavalin A (Sigma, St. Louis, MO) was added to a cell culture at a final concentration of 25 μg/ml. After an incubation of 5 min at room temperature, cells were washed twice with culture medium, further incubated in culture medium for 30 min, and harvested for preparation of total DNA and RNA.

DNA analysis. The relative abundances of endogenous α- and transfected α1 tubulin mRNA and Tuba1-st and Tuba1-lt minichromosomes were monitored by competitive PCR using DNA oligonucleotides Tub1-11 and Tuba1-20 (33) and total DNA prepared 5 weeks after transfection, a time that corresponds to approximately 30 generations. PCR products were separated by denaturing polyacrylamide gel electrophoresis (PAGE), visualized by silver staining, and quantified by densitometry.

RNA analysis. Total RNA was isolated from cells 3 weeks after transfection as described previously (33). Endogenous α- and transfected α1 tubulin mRNA and Tuba1-st and Tuba1-lt RNAs were analyzed by primer extension of the 5′-end 32P-labeled oligonucleotide Tub1-11 using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the recommendations of the manufacturer. Primer extension products were separated on 6% polyacrylamide–50% urea gels, visualized by autoradiography, and quantified by densitometry.

For determination of the TISs of the S. lemnæ α1 tubulin, α2 tubulin, β1 tubulin, β2 tubulin, cathepsin L, and Hsp70 minichromosomes, 5′-end 32P-labeled oligonucleotides complementary to the 5′-terminal regions of the protein-coding regions were used in both primer extension reactions of total RNA preparations and linear amplification sequencing reactions of plasmids harboring the complete minichromosomal sequence. Sequencing reactions were conducted with the delta Taq cycle sequencing kit (U.S. Biochemicals, Cleveland, OH) by following the manufacturer’s protocol. The resulting sequencing ladders were separated adjacent to the corresponding primer extension products by denaturing PAGE, which allowed unambiguous TIS identification on autoradiographs.

RESULTS

Expression of two transfected and differently tagged α1 tubulin minichromosomes. As with most minichromosomes of stichothricine ciliates, the S. lemnæ α1 tubulin minichromosome harbors a single protein-coding region and short noncoding flanks on either side (Fig. 1A). The 5′ flank consists of 20 bp of telomeric repeats, 155 bp of nontranscribed sequence, and a 37-bp transcribed but untranslated region. For the analysis of minichromosome replication, we have recently established stable transfection of two differently tagged α1 tubulin minichromosomes into vegetatively growing S. lemnæ (33). The Tuba1-st minichromosome has a 19-bp unrelated tag sequence inserted at position 279 relative to the 5′ end of the minichromosome’s noncoding strand; in the Tuba1-lt minichromosome, this tag is extended to a total of 49 bp (33). A key feature of both tags is the presence of several translation stop codons, which prevents the expression of aberrant and toxic α-tubulin molecules (33; also data not shown). By using DNA oligonucleotides Tub1-11 and Tub1-20, which hybridize to either side of the tags, the relative abundances of Tuba1-st, Tuba1-lt, and endogenous α1 tubulin minichromosomes could be monitored by competitive PCR (Fig. 1B). As has been shown previously, this assay produced results congruent to those of Southern blot analysis (32, 33). In our initial study, we found that the relative amount of transfected DNA decreased during the first generations after injection but became stable within 21 days, or approximately 30 cell divisions (32). Despite this decline, when an equimolar mix of Tuba1-st and Tuba1-lt minichromosomes was injected, we observed very similar ratios of these two DNAs once a stable cell line was obtained (Fig. 1C (33). To evaluate α1 tubulin expression from these minichromosomes, we prepared RNA from a transfected cell line and employed a primer extension assay using the end-labeled oligonucleotide Tub1-11, which hybridized to endogenous α-tubulin mRNA, to Tuba1-st RNA, and to Tuba1-lt RNA (Fig. 1D). Due to the tag sequences, the extension products of the two latter RNAs were distinctly longer, allowing evaluation of the expression level from each of the introduced minichromosomes. Importantly, the Tuba1-st/Tuba1-lt signal strength ratios from DNA and RNA analyses were consistent (Fig. 1C and D, compare st and lt signal strengths). This was also true when cell lines that contained significantly different levels of transfected DNA in comparison to endogenous α-tubulin DNA were investigated, strongly suggesting that the RNA levels were determined mainly by minichromosome abundance and were not subject to posttranscriptional regulation (data not shown). We therefore concluded that this cotransfection system is suitable for promoter characterization.

Expression effects of α1 tubulin minichromosome 5′ deletions. In a first set of experiments to characterize the α1 tubulin minichromosome promoter, we progressively deleted the 5′ nontranscribed region of the Tuba1-st minichromosome (Fig. 2A). Mutated Tuba1-st and unmodified Tuba1-lt minichromosomes were mixed in equimolar amounts, and the mixture was microinjected into the macronuclei of individual S. lemnæ cells. A minimum of three clonal cell lines derived from independent microinjections were raised for each mutation. The Tuba1-st/Tuba1-lt DNA and RNA ratios in these cell lines were then determined by quantification of competitive PCR and primer extension signals, respectively (Fig. 2B). As expected, the full-length 5′ flank resulted in an expression ratio of approximately 1. Interestingly, deletion of the first 41 bp consistently resulted in increased Tuba1-st expression (Fig. 2B, del1). This expression enhancement may have been caused by a change of nucleosome positioning or the deletion of a negative regulatory sequence. Deleting the 5′ flank further, to position −55 relative to the TIS, reduced Tuba1-st expression to 10%, suggesting the presence of an important upstream sequence element in the α1 tubulin minichromosome promoter (Fig. 2B, del2). While a deletion to position −35 caused a comparable result, Tuba1-st expression was virtually abolished.
when the nontranscribed region was deleted to position −10, suggesting that the promoter contained an essential sequence element within the 35-bp region upstream of the TIS (Fig. 2B, del3 and del4). In sum, this initial analysis indicated that the α1 tubulin minichromosome promoter consisted of an essential proximal promoter and an upstream activation region.

**Mapping of promoter elements by block substitution analysis.** To identify individual promoter elements, we carried out block substitution analysis for the region upstream of the TIS from position +1 to position −95 (Fig. 3A). Three distinct promoter elements were found. The most proximal element was identified by mutation sub8, which extended from position −20 to position −27, comprising the wild-type nucleotides AAATACTT (Fig. 3A and B). If the T residue at position −28 is included, the corresponding motif TAAATA is reminiscent of the classical TATA box TATAAA (34). This box is a conserved class II core promoter element in eukaryotes, and by binding the TATA-binding protein or the general transcription factor IID, it is involved in recruiting RNA polymerase II to the correct TIS. Accordingly, mutation of this element in the α1 tubulin promoter caused partly aberrant transcription initiation (Fig. 3B, sub8). Hence, we termed this sequence the TATA-like element.

The next distinct promoter element further upstream was identified by the sub5 mutation, which extended from position −44 to position −52. This mutation reduced Tuba1-st expression by 83% but did not cause aberrant initiation, indicating that this upstream sequence element 1 (USE1) is involved in transcription activation (Fig. 3B, sub5). The corresponding sequence motif CTCATATC has no obvious similarity to known promoter elements. Interestingly, in an early comparative analysis of putative *T. thermophila* promoters, the only conserved sequence identified was TATCCAATCARA, indicating the presence of CAAT boxes in ciliate promoters (3). In the same study, a corresponding sequence element with one mismatch was found in the 5′ flank of the *S. lemnæ* α1 tubulin minichromosome. In sub5, the first four nucleotides of this motif are mutated, which could possibly account for the observed effect. On the other hand, mutation of the remainder of this motif, including the putative CAAT sequence in sub6,
reduced the expression efficiency only moderately, to 46%. Hence, a more detailed analysis will be required to determine which of these residues are important for binding of transcriptional trans-activators. The two adjacent block substitutions sub2 and sub3 diminished Tuba1-st expression similarly to sub5 (Fig. 3B) and therefore identified USE2 in the region between positions −67 and −85 (Fig. 3A). Interestingly, deletion or mutation of either USE reduced transcription efficiency similarly, between 80 and 90%. The effects of deleting only USE1 or both USEs were not significantly different (Fig. 2B, del2 and del3). These results therefore suggest that these two USEs function cooperatively in activation of α1 tubulin minichromosome transcription.

The TATA-like element appears to be generally present in minichromosomes of stichotrichs. Only very few transcription initiation sites have been mapped in S. lemnae thus far, and in most cases the mapping results did not unambiguously reveal the initiation nucleotide (32). We therefore revisited and identified the TISs from the S. lemnae α1, α2, β1, and β2 tubulin as well as those from the Hsp70 and cathepsin L minichromosomes. When the sequences were aligned according to the TIS, it became apparent that 6-residue AT motifs starting at positions −30 to −28 are present in all these sequences (Fig. 4). This finding is in close agreement with the findings for TATA boxes of mammalian and insect genes, which are located between 25 and 30 bp upstream of the TIS (reviewed in reference...
Moreover, in a recent study of the related stichotrich Sterkiella histriomuscorum, the TISs of 10 minichromosomes were determined by cDNA characterization (19), and alignment of the corresponding upstream sequences revealed the same AT motifs at the predicted position (data not shown). Only in one of these elements was the motif disrupted by a single C residue (data not shown). The AT motifs of the S. lemnae and S. histriomuscorum minichromosomes do not ex-

![Diagram](https://example.com/diagram.png)

**FIG. 3.** Mapping of α1 tubulin minichromosome promoter elements. (A) Alignment of the wild-type sequence upstream of the TIS and those of Tub1-st block substitution mutants. Promoter sequences whose mutation reduced Tub1-st expression by more than 80% are boxed. Dots represent identical nucleotides. (B) DNA and RNA analysis as described in the legend to Fig. 2B. The asterisk marks a primer extension band indicative of an aberrant TIS.

![DNA and RNA Analysis](https://example.com/diagram.png)

**FIG. 4.** Alignment of core promoter sequences of S. lemnae minichromosomes. TISs of the α1, α2, β1, and β2 tubulin (tub) minichromosomes, as well as those of the cathepsin L (Cath) and Hsp70 minichromosomes, were determined by a combination of primer extension assays and linear amplification sequencing. The sequences are aligned without gaps according to the TIS. The AT motifs starting at positions −30 to −28 are shaded in gray. Invariant and W positions of the putative initiator sequences are shaded in black and gray, respectively. In addition, an invariant A residue at position −6 is shaded in black. The asterisk indicates the TIS (+1). The bar indicates the sub4 mutation, which extends over the TATA-like element.
hibit a clear consensus sequence, and further functional analysis will be required to determine the importance of single residues within these motifs. This apparent lack of conservation, however, is in accordance with the finding that a wide variety of AT-rich sequences can function as TATA boxes in other eukaryotes (10, 28, 31), especially when another strong core promoter element is present, e.g., an initiator (24, 39). Taken together, these data suggest that a TATA-like motif starting approximately at position −30 is a general promoter element in the minichromosomes of stichotrichous ciliates. Since TIS mapping and a comparison of upstream sequences revealed putative TATA-like elements around position −30 in macronucleus minichromosomes of the hypotrichous ciliate *Euplotes crassus* (8), it is possible that a TATA-like element is a common component of ciliate gene promoters.

**Evidence for an initiator element.** The alignment in Fig. 4 shows that, besides the AT motifs, the sequence surrounding the TIS is conserved, with a consensus of 5′-WWWTAT (+1)ANT-3′. In *S. histrio muscorum*, the corresponding consensus is very similar, 5′-WNWTAA (+1)WTW-3′, if the aberrant TIS sequence of the permease minichromosome is omitted (19; also data not shown). Moreover, initiator elements have been described for both protistan and higher eukaryotes, and the TIS within the initiator element invariably is an adenine residue (16, 23, 34). In accordance with these findings, transcription of the analyzed *S. lemnæ* and *S. his trium scorum* minichromosomes initiates exclusively at an adenine residue (Fig. 4) (19). Taken together, these data indicate that an initiator element is a common and conserved element in promoters of stichotrichous minichromosomes. In agreement with the presence of an initiator in the *S. lemnæ* α1 tubulin minichromosome, mutation of the sequence from position −4 to position −11 did not affect the accuracy or efficiency of transcription (Fig. 3B, sub10), whereas mutation downstream of position −4 virtually abolished transcription (Fig. 3B, sub11). Moreover, TATA boxes and initiator elements function similarly, and both can direct RNA polymerase II to the correct transcription initiation site independently. For humans, both promoter elements have been shown to function synergistically when separated by 25 to 30 bp but to act independently when separated by more than 30 bp (27). In comparison, mutation of the TATA-like element in the *S. lemnæ* α1 tubulin minichromosome did not abolish transcription completely, and both aberrant transcription initiation and accurate transcription initiation were observed (Fig. 3, sub8). This result demonstrated that the TATA-like element was not absolutely required for the recruitment of RNA polymerase II to the correct TIS and that another element, presumably the initiator element, can in part assume this task. TIS mapping of the sub8 minichromosome revealed three sites, at positions +1, −2, and −6 (Fig. 5). The strongest TIS was at position −6. Interestingly, the sequence surrounding this site is a perfect repeat of the sequence around the correct TIS, confirming the notion that this sequence constitutes an initiator element which, in combination with the TATA-like element, determines transcription initiation.

**Transcriptional up-regulation of α-tubulin expression following concanavalin A treatment.** Our analysis of the α1 tubulin minichromosome promoter revealed a TATA-like element and evidence for an initiator element, both of which represent classical class II core promoter elements. In addition, we found two USEs important for activating transcription (Fig. 6A). Interestingly, the nontranscribed upstream region of the α2 tubulin minichromosome is very short; it extends only to position −36 and comprises an identical AT motif and the putative initiator element but no USEs (Fig. 6A and E). Due to the latter finding, we predicted that transcriptional regulation is confined to the α1 tubulin promoter and that α2 tubulin promoter transcription is constitutive. In contrast to this prediction, treatment of *S. lemnæ* cells with the plant lectin concanavalin A, which stimulates the expression of α tubulin, among others (22, 38), resulted in a predominant increase in α2 tubulin mRNA levels (38; also data not shown). To assess whether the α2 tubulin promoter is responsible for this effect, we constructed the chimeric minichromosome Tuba1-st-p2 by replacing the 5′ nontranscribed region of Tuba1-st with its 36-bp α2 tubulin counterpart (Fig. 6B). An equimolar mixture of the artificial minichromosomes Tuba1-lt and Tuba1-st-p2 was used to generate two stable and clonal cell lines. Although these cell lines maintained different copy numbers of transfected minichromosomes, the numbers of lt and st minichromosomes were nearly identical in each (Fig. 6C). In cell line 1, the expression from each injected minichromosome was as strong as that of endogenous α-tubulin, whereas in cell line 2, the expression of introduced minichromosomes was considerably lower (Fig. 6D, compare lanes 3 and 5). In both cell lines, the α1 tubulin promoter minichromosome Tuba1-lt was expressed with an approximately 30% higher efficiency than the α2 tubulin promoter minichromosome (Tuba1-st-p2), suggesting that constitutively α1 is the stronger promoter (compare st and lt signals in Fig. 6C and in lanes 3 and 5 of Fig. 6D). Independently of the expression level, however, the relative abundance of st RNA was increased approximately fourfold 30 min after concanavalin A treatment (Fig. 6D, compare lanes 3 and 5).
and 5 with lanes 4 and 6, respectively). Since this effect is based exclusively on the nontranscribed upstream sequence, it can be concluded that Tubal-st-p2 expression under these conditions was up-regulated through transcriptional activation. The activation was not dependent on a positional effect, e.g., the position of the telomere relative to the TIS, because deletion of the α1 tubulin minichromosome promoter to position −36 resulted in a decrease in expression comparable to that of the del2 and del3 deletions (Fig. 2) and could not be induced by concanavalin A (data not shown). Thus, the regulatory determinant must reside within the 36-bp α2 tubulin minichromosome promoter. Since the AT motif and the preceding sequence of the TIS are identical for the α1 and α2 tubulin promoters, it is likely that α2-specific residues form an additional regulatory element (Fig. 6E). In sum, the results presented here demonstrate that stichotrichous ciliates utilize different promoter structures to regulate gene expression at the level of transcription initiation.

DISCUSSION

In this study, we have functionally characterized the S. lemanae α1 tubulin minichromosome promoter in vivo by a mutational analysis of the 5′ nontranscribed spacer. As core promoter elements, we identified a critical sequence around the TIS that appears to be an initiator element and a TATA-like...
element around position −25. While mutation of the TATA-like element caused aberrant transcription initiation, mutation of the sequence surrounding the TIS virtually abolished transcription, indicating that the TATA-like element and the initiator are core promoter elements that cooperate in the recruitment of RNA polymerase to the correct TIS. The 5′ nontranscribed spacers of stichotrichous ciliates are highly AT rich (14), but accurate mapping of TISs in other S. lemnæ minichromosomes allowed a correct assignment of sequence elements according to their positions relative to the TIS. Taking the positional information into account, it appears that the TATA-like element and initiator form a conserved bipartite core promoter in S. lemnæ minichromosomes. Since TIS mapping and sequence comparison revealed highly similar sequence elements in the related stichotrich S. histriomuscorum (reference 19 and this study), it is likely that the bipartite core promoter structure is generally present in minichromosomes of stichotrichous ciliates.

Besides the TATA box and initiator, eukaryotic core promoters may contain a downstream promoter element (4), a downstream core element (20), or a motif ten element (21). In our in vivo analysis, we omitted mutations downstream of the TIS, because they may have affected RNA stability rather than transcription efficiency. We therefore cannot rule out the presence of downstream components in the S. lemnæ α-tubulin minichromosome promoters. However, the presence of such elements is unlikely, because they have been found only in insects and mammals thus far, and they most often occur in TATA-less promoters (reviewed in reference 34).

In addition to the core promoter, the α1 tubulin minichromosome harbors two USEs, which seem to form a bipartite activator element. Searches with the USE sequence did not reveal a conserved motif in the 5′ nontranscribed spacers of other S. lemnæ minichromosomes (data not shown), suggesting that these USEs either are specific to the α1 tubulin minichromosome or require only a few conserved residues for trans-activator binding. At the current stage, the functional significance of the USEs is not understood. Whereas they are clearly needed for maximal α1 tubulin minichromosome expression, the short α2 tubulin minichromosome upstream of the TIS promoted nearly the same level of transcription in the α2/α1 hybrid minichromosome Tuba1-st-p2 (this study), and results from a separate study investigating endogenous α-tubulin transcript levels and stability even suggested that the α2 tubulin promoter is stronger than its α1 tubulin counterpart (38). Moreover, it was the α2 and not the α1 tubulin promoter that was activated upon induction of α-tubulin gene expression by concanavalin A treatment (this study). These findings, however, do not rule out the involvement of the USEs in α-tubulin expression regulation. Currently, we do not know the significance of expressing two slightly different α-tubulins. Thus, different stimuli may act differently on these promoters. Alternatively, the USEs may be involved in life cycle-specific regulation of α-tubulin expression.

Macronuclei of ciliates contain gene-specific copy numbers of minichromosomes. For example, a single S. lemnæ cell harbors approximately 150,000 copies of the α1 tubulin minichromosome (38) but only about 3,500 copies of the calmodulin minichromosome (7). It is therefore possible that gene dosage is a major determinant of gene expression in ciliates (17). On the other hand, our results and data obtained from different ciliates demonstrate that this group of organisms is quite capable of regulating gene expression at the level of transcription initiation. One striking example is the TATA-binding protein minichromosome in E. crassus, which is transcribed at a 160-fold-higher rate than the β-tubulin minichromosome (2). Another example is the promoter of the metallothionein gene of T. thermophila, which is silent or highly active in the absence or presence of cadmium cations, respectively (30). To understand transcription regulation in ciliates, promoter elements need to be characterized and trans-acting factors identified. Our study represents an important step in this direction.

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