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Widespread Use of TATA Elements in the Core Promoters for RNA Polymerases III, II, and I in Fission Yeast

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In addition to directing transcription initiation, core promoters integrate input from distal regulatory elements. Except for rare exceptions, it has been generally found that eukaryotic tRNA and rRNA genes do not contain TATA promoter elements and instead use protein-protein interactions to bring the TATA-binding protein (TBP), to the core promoter. Genomewide analysis revealed TATA elements in the core promoters of tRNA and 5S rRNA (Pol III), U1 to U5 snRNA (Pol II), and 37S rRNA (Pol I) genes in *Schizosaccharomyces pombe*. Using tRNA-dependent suppression and other in vivo assays, as well as in vitro transcription, we demonstrated an obligatory requirement for upstream TATA elements for tRNA and 5S rRNA expression in *S. pombe*. The Pol III initiation factor Brf is found in complexes with TFIIIC and Pol III in *S. pombe*, while TBP is not, consistent with independent recruitment of TBP by TATA. Template commitment assays are consistent with this and confirm that the mechanisms of transcription complex assembly and initiation by Pol III in *S. pombe* differ substantially from those in other model organisms. The results were extended to large-rRNA synthesis, as mutation of the TATA element in the Pol I promoter also abolishes rRNA expression in fission yeast. A survey of other organisms' genomes reveals that a substantial number of eukaryotes may use widespread TATAs for transcription. These results indicate the presence of TATA-unified transcription systems in contemporary eukaryotes and provide insight into the residual need for TBP by all three Pols in other eukaryotes despite a lack of TATA elements in their promoters.

Structural similarities shared by the RNA polymerases (Pols) of bacteria, archaea, and eukarya reflect a deep-rooted common ancestry of transcription systems in all organisms on earth. Archaea and eukaryotes exhibit greater similarity to each other in their Pol subunits, accessory transcription factors (TFs), and promoter elements than either does to bacteria (36). In eukaryotes, Pol I synthesizes large rRNA (35S to 45S, depending on the species); Pol II synthesizes mRNAs and some small nuclear (sn) RNAs, such as U1 to U5; and Pol III synthesizes mostly tRNAs and 5S rRNA, as well as U6 snRNA and a few other transcripts (53).

The core promoter orchestrates polymerase recruitment, promoter activity, and response to regulatory input (59, 66). In eukaryotes, TATA promoter elements direct transcription by Pol II of a large subset of (but not all) protein-encoding genes, but often not the far fewer snRNA genes that are transcribed by Pol II. While TATA elements are found in a minute fraction of Pol III genes, they are generally not found in the core promoter regions of Pol I genes (53). Intriguingly, despite the lack of TATA promoter elements, Pols I, II, and III all require TATA-binding protein (TBP) for initiation (17). Archaea use widespread TATA-like promoters and a TBP ortholog to direct transcription by a single Pol of all gene types, those encoding tRNA, rRNA, and mRNAs (reviewed in reference 67). Orthologs of another central initiation factor, TFIIB, cooperate with TBPs in promoter recognition in archaea and eukarya (35, 36). While TBP is shared by the three eukaryotic Pols, TFIIB and related factors exhibit polymerase and promoter specificity, such that TFIIB is used by Pol II, TFIIB-related factor (Brf) is used by Pol III for tRNA and 5S rRNA genes, and a distinct variant, BRFU/TFIIIB50, is used by Pol III for human U6 and related type 3 genes (63, 70). The TFIIB-related proteins bind adjacent to TBP on the promoter, recruit the corresponding polymerase to the transcription start site, and participate in promoter melting, an intermediate step in initiation (27, 30, 51, 58). Unlike archaeal and the eukaryal Pol II and Pol III systems, there is no apparent TFIIB homolog in the Pol I machinery (11, 84). Instead, the factor known as Rrn3p/TIF-IA bridges the core promoter-associated factors and Pol I (2, 44, 54).

Pol III promoters have historically been categorized into three major types. 5S rRNA (type 1) and tRNA (type 2) genes utilize internal TATA-less promoters, whereas U6 snRNA (type 3) promoters contain upstream TATA elements (7, 15, 28, 53, 80). For TATA-containing genes such as U6, TBP-TFIIIB can recognize the upstream DNA directly (46, 78). The tRNA promoter is composed of a proximal A box element located 10 to 20 bp downstream of the start site of transcription and a B box element at various distances farther downstream. Although the regions upstream of eukaryotic tRNA genes are generally AT rich, the sequence in this region is not conserved (33). Rather, the sequence information used to assemble a tRNA transcription complex resides in the internal promoter, which is recognized by TFIIIC. Once bound, TFIIIC recruits the initiation factor Brf and its associated TFIIIB components to the TATA-less upstream DNA (7, 28, 80). TFIIIB is an

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entity composed of three polypeptides, TBP, Brf, and B″ (7, 28). TBP is brought to the upstream region of the tRNA gene by Brf (designated BRF/hTFIIIB90 in the human system) via stable protein-protein interactions that occur in the absence of DNA (22, 31, 75). Therefore, association with Brf provides TBP access to the TATA-less tRNA promoter (31, 40, 41, 77). In this setting, TATA is not required and the TBP in TFIIIB can bind to upstream DNA that contains stretches of only G and C residues (25).

We discovered that TATA motifs reside upstream of nearly all *Schizosaccharomyces pombe* tRNA and 5S rRNA genes. Here we demonstrate an obligatory role for TATA in homologous 5S rRNA and tRNA expression in *S. pombe*. We demonstrate differences in the mechanisms of Pol III transcription complex formation in *S. pombe* and *Saccharomyces cerevisiae* using in vitro transcription systems. Furthermore, *S. pombe* Brf associates with TFIIIC and Pol III in vivo, while TBP is conspicuously absent from these complexes, consistent with a TATA-dependent mechanism of TBP recruitment. The cumulative data fit a model of obligatory recruitment of TBP by the TATA element, precluding a stringent need for Brf-mediated recruitment of TBP, and lead to the proposal that this may reflect an ancient Pol III system.

Widening our search revealed TATA elements upstream of the *S. pombe* genes for U1 to U5 snRNAs and ≈37S rRNA genes, which do not use TATA elements in several other species. Mutation of the TATA element in the Pol I core promoter abolishes rRNA expression in vivo. The data indicate that all three Pols require TATA promoter elements for efficient expression in fission yeast. These results suggest that *S. pombe* may represent an ancient eukaryotic transcription system that was intermediate between that in archaea and the more diversified eukaryotic model systems that are described in textbooks.

MATERIALS AND METHODS

Construction and expression of TATA-less tRNA, 5S rRNA, and ≈37S rRNA genes and *F-Ret1*. The *S. cerevisiae* $tRNA^{ser}$ gene (on chromosome IX) was amplified from genomic DNA, cloned into the pJK vector, and named $SctRNA^{ser}UCA$. TATA was then introduced at −30; this changed the upstream sequence TATCTACAA to TATATATAA. Plasmid pFL20/18SPst5.8Si4, containing full-length *S. pombe* ribosomal DNA (rDNA) and 500 bp of upstream sequence producing a sequence-tagged 5.8S* rRNA (23), was left unaltered or changed at the TATAAA sequence to GGATCC by site-directed mutagenesis to create pFL20/18SPst5.8Si4-Mu2-6 (and -Mu2-7). The plasmids were used to transform *S. pombe* strain yAS50, and transformants were selected and grown on Edinburgh minimal medium lacking uracil. The 5S rDNA gene with flanking sequences (43) was amplified by PCR from *S. pombe* DNA and cloned into pGEM-T (Promega, Madison, Wis.) to create p5S-T, which was mutagenized at four nucleotide positions to create plasmid p5Smu. The 5Smu-containing fragment was subcloned into the *Nco*I/*Nde*I sites of pRep90X (21) to create pRep90X-5Smu. The TATA element of p5Smu was mutagenized and cloned into the *Nco*I/*Nde*I sites of pRep90X to create pRep90X-5Smu-Bam. These were used to transform yAS50, and transformants were selected on EMM lacking leucine. $Ret1^+$ was amplified from genomic DNA. The product was cloned into the *Sal*I and *Sma*I sites of pREP3X, resulting in pREP3X-Ret1, which was digested with *Pst*I and *Bam*HI and cloned into pBluescript-ura4, resulting in pBluescript-Ret1a. *Ret1* 5′-flanking sequence was obtained by PCR of *S. pombe* genomic DNA. The product was cloned into the *Kpn*I and *Sal*I sites of pBluescript-Ret1a, resulting in pBluescript-Ret1b. A 4.2-kb *Kpn*I/*Bam*HI fragment of pBluescript-Ret1b was transformed into yHL6382 (21) to create yYH3272 (h^+ *his3-D1 leu1-32 ura4-D18 ade6-M216* Δ*ret1*::[*F-ret1*, $ura4^+$]). The genomic structure of *F-ret1* in yYH3272 was confirmed by PCR (not shown). All constructs were verified by sequencing.

Immunoaffinity purification of Pol III and TFIIIC complexes. Extracts were prepared according to the method of Hamada et al. (16) with modifications. Cells were broken with a French press two times, and the lysate was prepared by adding 3.5 M $(NH_4)_2SO_4$ to a final concentration of 0.4 M. The resulting precipitate was dissolved in BC100 (20 mM HEPES, pH 7.9, 20% glycerol, 0.5 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 100 mM KCl) and dialyzed into BC100. For immunoaffinity purification, the extract was adjusted to 0.05% NP-40. Two milligrams of extract protein was incubated with 40 μl of M2 agarose beads (Sigma) at 4°C for 4 h. The beads were washed five times with 0.5 ml of BC100) 0.05% NP-40) and eluted two times with 40 μl of BC100 containing 200 μg of FLAG peptide (Sigma)/ml.

Antisera and immunoblotting. The following antigens were used for antiserum production: N-terminal peptides of Ret1p, spB″, and Rpc39p, and a C-terminal peptide of spBrf. Anti-TBP, anti-Sfc1p, ant-Sfc3p, anti-Sfc6p, anti-Sfc4p, and anti-spLa (Sla1p) were described previously (21). Samples were separated by 4 to 20% polyacrylamide gel electrophoresis, transferred to nitrocellulose, probed with appropriate antibodies, and processed using an ECL kit (Amersham).

RESULTS

TATA elements upstream of *S. pombe* tRNA genes. For this study, tRNAscan-SE (42) was used to identify the tRNA gene locations, and then the upstream regions were extracted from *S. pombe*, *S. cerevisiae*, *Homo sapiens, Drosophila melanogaster*, and *Arabidopsis thaliana* genomic databases. The sequence sets were then used to generate sequence Logos (Fig. 1A) (62). In the Logo display, the bases are stacked on top of each other for each position, the height of each letter being proportional to its frequency, and the letters are sorted with the most common one on top. The height of each stack is adjusted according to the information content (i.e., conservation) and plotted, in bits, on the base 2 logarithmic scale on the *y* axis (62).

Figure 1A shows the upstream sequence (the first base of the mature tRNA is designated M1 under the *A. thaliana* Logo) and the first half of the tRNA sequence (ending after position 37), followed by a gap representing introns and sequences extending through the variable stem-loop (not shown), followed by the B box region and continuing to the end of the tRNA sequence (CCA is not included). The positions of the A box and B box and some features of tRNA structure are indicated. The largest amount of information content coincides with the A and B box promoter elements. In the upstream regions, the information content is higher for *S. pombe* than for the other species. The most concentrated upstream information appears as a TATAAA motif at a position typical of TATA promoter elements (recall that Pol III initiation occurs a variable distance [≈5 to 10 bp] upstream of the first base of the mature tRNA). This degree of information was not found in the upstream regions of the *S. cerevisiae*, *H. sapiens*, and *D. melanogaster* tRNA sequences (not shown, but see below). TATA-like information also appeared upstream of *A. thaliana* tRNA genes, although the information content was not as high nor as well defined as for *S. pombe* (Fig. 1A), consistent with the documented presence of the 4-nucleotide sequence TATA (13).

tRNAscan-SE used the first base of the mature tRNA sequences to isolate the upstream sequences. However, Pol III initiates at various distances (≈5 to 15 bp) upstream of the mature tRNA sequence. Prealignment of the sequences by an alignment program led to a significant increase in the information content of the TATA region in the *S. pombe* and, to a lesser degree, *A. thaliana* sequences (Fig. 1B) but not those of the other species (not shown).

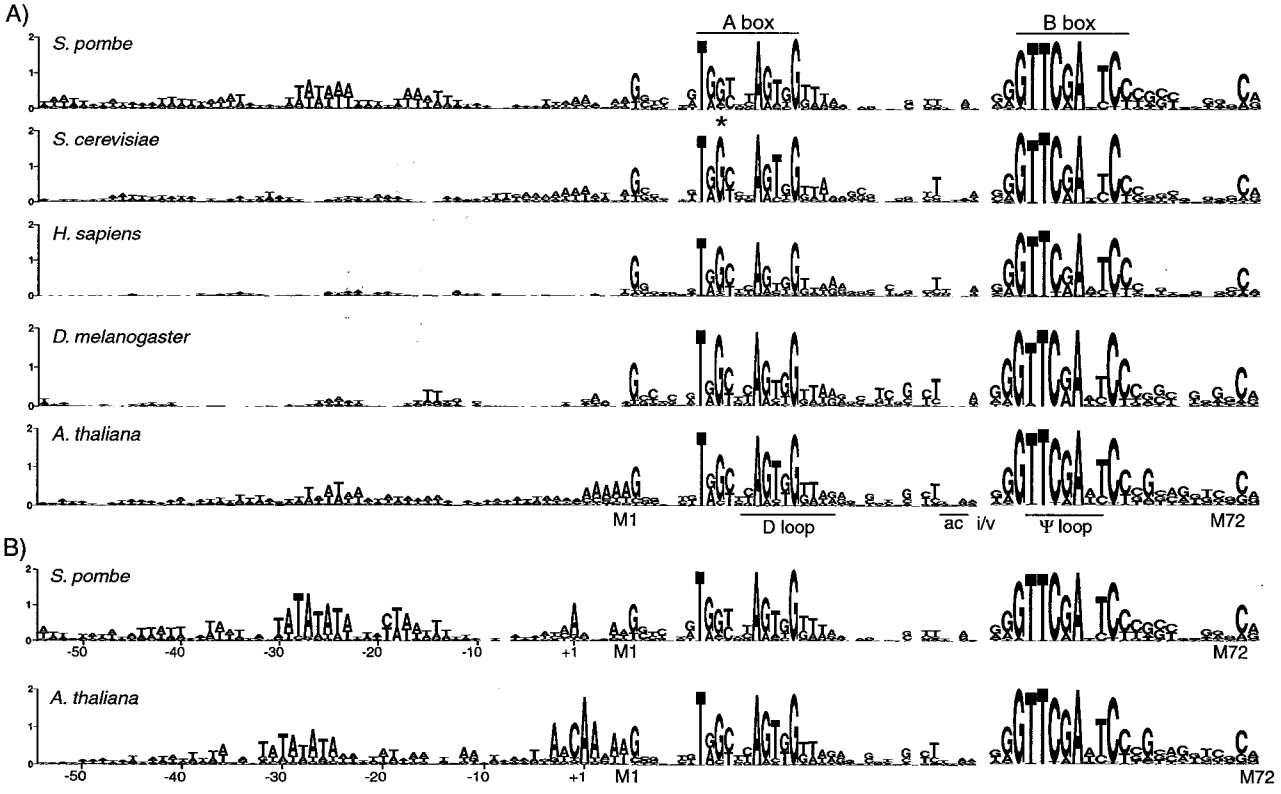

FIG. 1. Genomewide analysis reveals TATA motifs upstream of *S. pombe* tRNA genes. (A) Sequence Logos of the genomic 5′ flanking regions of tRNA sequences at 174 *S. pombe*, 275 *S. cerevisiae*, 425 *D. melanogaster*, 625 *H. sapiens*, and 600 *A. thaliana* loci. The positions of the A and B box promoter elements are indicated above the Logos. Note the relatively low level of sequence information content in the *S. pombe* A box, at the no. 10 position indicated by the asterisk, compared to that of *S. cerevisiae*. Although the full sets of sequences were used for the upstream regions and the first half of the tRNA for all species, for *H. sapiens*, *A. thaliana*, and *D. melanogaster*, the B box and downstream regions were limited to 200, 215, and 250 sequences, respectively, for practical reasons. Approximately 20% of the *H. sapiens* sequences were identified by tRNAscan-SE as "possible pseudogenes," probably reflective of tRNA-like repetitive elements in the human genome (42). Approximately 1% each of the *A. thaliana*, *S. pombe*, and *D. melanogaster* sequences and none of the *S. cerevisiae* sequences were identified as possible pseudogenes. Common features of tRNA structure are indicated below the Logos. M1 indicates the first base of mature tRNA. A gap representing introns and sequences extending through the variable stem-loop (not shown) is designated i/v. ac, anticodon. (B) Sequence Logo of the *S. pombe* and *A. thaliana* sequences after prealignment of the upstream regions by the Clustal program. The numbering under the upstream region is relative to the putative consensus +1 site.

To our surprise, prealignment also produced a prominent A that appeared in the *A. thaliana* and *S. pombe* sequences about six positions upstream of the mature tRNA (Fig. 1B). An increase in the information content of this position in association with the increased information content of the TATA motif that appeared in the aligned sequences (more so for *S. pombe* than *A. thaliana*), in conjunction with the 30-bp spacing of the prominent A and TATA, argued that the A represents a consensus transcription initiation site (+1). While prealignment led to a moderate increase in the TATA content of the *A. thaliana* sequences, a more substantial increase was observed around the predicted +1 site (underlined), which appeared in the form of ANCAA (Fig. 1B). ANCAA may be analogous to the initiator element described for certain Pol II (Inr) and Pol I (rInr) core promoters (56, 66).

Inspection of individual *S. pombe* sequences revealed that while 35% matched at seven or more TATATATA positions, the great majority matched at five or more positions (not shown). Thus, most, if not all, *S. pombe* tRNA genes appear to have at least a component of an upstream TATA element around the −30 region (not shown). Our ability to examine tRNA transcription in fission yeast led us to focus on *S. pombe* for the remainder of this study.

G10, which occupies the third position of the A box and is invariant in all *S. cerevisiae* tRNA genes and highly conserved in those of other species (14, 15), is much less conserved in *S. pombe* (Fig. 1A). Analysis of the subset of the *S. pombe* non-G10 genes revealed that they were similar to the full set of sequences in several features, including an upstream TATA, A-to-B box distance, percentage of genes with introns, +1 information, and B box consensus (not shown). We conclude that upstream TATAs are typical of the great majority of *S. pombe* tRNA genes. As will be described in more detail below, template commitment and other in vitro transcription assays using *S. pombe* extract indicate that although tRNA genes with G10 appear to compete better than non-G10 genes for a limiting TF (probably TFIIIC [see below]), these nonetheless require upstream TATA elements regardless of whether G occupies the number 10 position, i.e., even when the A and B boxes exhibit perfect matches to the *S. cerevisiae* consensus.

TATA promotes tRNA expression in *S. pombe* and in a homologous in vitro transcription system. We recently characterized an in vivo suppression assay that is sensitive to the expression level of $tRNA^{ser_{UGA}}$ (16). Two suppressor genes were described, $tRNA^{ser_{UGA\text{-}W}}$, which encodes a wild-type full-strength suppressor, and$tRNA^{ser_{UGA\text{-}M}}$, which is comparably active for Pol III transcription but is less active for suppression (16). The naturally occurring TATATAAA sequence was altered in both of these genes, and their suppressor activities in *S. pombe* were determined (Fig. 2A). While both genes containing the wild-type TATA were active (16), the TATA-less genes were inactive.

The effects of TATA on the efficiency of in vitro transcription in *S. pombe*-derived extract are shown in Fig. 2B. In addition to the *S. pombe* $tRNA^{ser_{UGA\text{-}M}}$ gene, we also examined an *S. cerevisiae* $tRNA^{ser}$ gene that contains perfect matches to the consensus A and B box promoter elements and, as is typical of *S. cerevisiae* tRNA genes, contains no upstream TATA element. Specifically, the A and B box elements in the two yeasts' $tRNA^{ser}$ genes used here are identical except for one position in the A box, T10 in the *S. pombe* $tRNA^{ser}$ gene and G10 in the *S. cerevisiae* $tRNA^{ser}$ gene. These genes were used for in vitro transcription (Fig. 2B). Nucleotides in the wild-type TATA-less *S. cerevisiae* $tRNA^{ser}$ gene were replaced to create a TATA element. The TATA-less *S. cerevisiae* gene was inactive, while the substitutions that created the TATA element activated it (Fig. 2B, lanes 1 and 2). This demonstrated that a yeast tRNA gene with perfect matches to the consensus A and B box elements requires an intact upstream TATA for efficient transcription in the *S. pombe* system. The *S. pombe* $tRNA^{ser_{UGA\text{-}M}}$ gene whose TATA was mutated was inactive, while the gene containing wild-type TATA was active (Fig. 2B, lanes 3 and 4). A negative control, showing the products of a reaction containing a control plasmid that does not contain a tRNA gene, is shown in lane 5, and another version of the TATA-containing $tRNA^{ser_{UGA\text{-}M}}$ gene that differs only in the sequence at the Pol III terminator and therefore produces a distinctively longer transcript (16) is shown in lane 6.

An essential TATA element upstream of 5S rRNA genes in *S. pombe*. 5S rRNA genes are dispersed in *S. pombe*, flanked by sequences that vary among copies (43). The upstream flanking regions plus the first G of the 5S rRNA sequences of multiple 5S rDNA loci are shown as a sequence Logo in Fig. 3A. Although variability is apparent at many positions, a TATA motif is prominent and typical for the majority of 5S genes.

To investigate if the upstream TATA is important for expression, we examined 5S rRNA production from a pRep90X-derived plasmid containing an *S. pombe* 5S rRNA gene (43). We introduced four substitutions in the 5S sequence that correspond to nonconserved residues (derived mostly from *S. cerevisiae* 5S). These substitutions allow specific detection by oligonucleotide hybridization that distinguishes expression from the test gene, designated 5S*, from that of endogenous 5S rRNA and are also compatible with the predicted 5S rRNA secondary structure in this region (not shown). The wild-type upstream TATATAA was then changed to GGATCCA or left unaltered, and the resulting plasmids, which differed only at the TATA sequence, were used to transform *S. pombe* to a selectable *leu1*$^+$ phenotype. Both plasmids yielded transformation efficiencies comparable to that of the empty control plas-

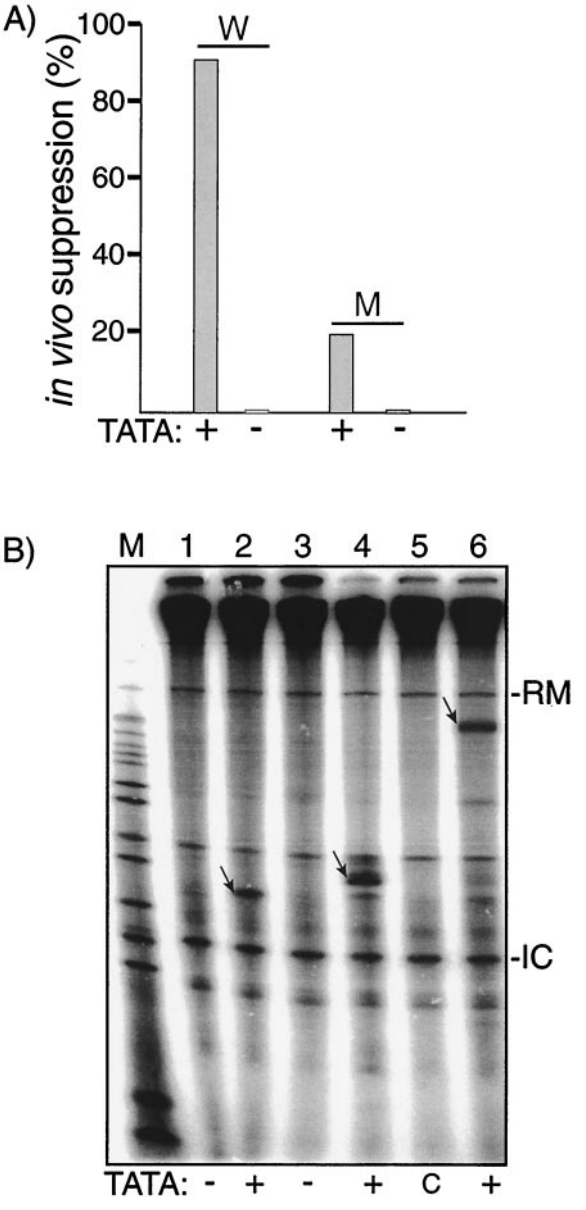

FIG. 2. Upstream TATA is a positive determinant of tRNA expression in vivo and in a homologous in vitro *S. pombe* system. (A) The TATATA motifs of two opal suppressor tRNA genes, $tRNA^{ser_{UGA\text{-}W}}$ (W) and $tRNA^{ser_{UGA\text{-}M}}$ (M), were left unchanged (+) or replaced with GGATCC (−) as indicated along the horizontal axis and examined for suppressor activity in vivo as previously described (16, 24). (B) In vitro transcription in an *S. pombe*-derived extract was performed using three tRNA genes, containing (+) or lacking (−) upstream TATA elements, and empty plasmid control (c) as indicated below the lanes. Lanes 1 and 2, *S. cerevisiae*-derived $tRNA^{ser_{UCA}}$ gene; lanes 3 and 4, *S. pombe*-derived $tRNA^{ser_{UGA\text{-}M}}$ gene; lane 5, control plasmid containing no tRNA gene; lane 6, *S. pombe*-derived $tRNA^{ser_{UGA\text{-}M\text{-}3T}}$ gene (produces longer transcript [16]). The *S. cerevisiae* and *S. pombe* genes differ in size due to a 15-nt intron in the latter, and their A and B box elements are identical except at one position, G10 in the former and T10 in the latter. Transcript bands are indicated by arrows. -RM indicates a recovery marker added to the reactions, and -IC indicates an extract-derived internal control.

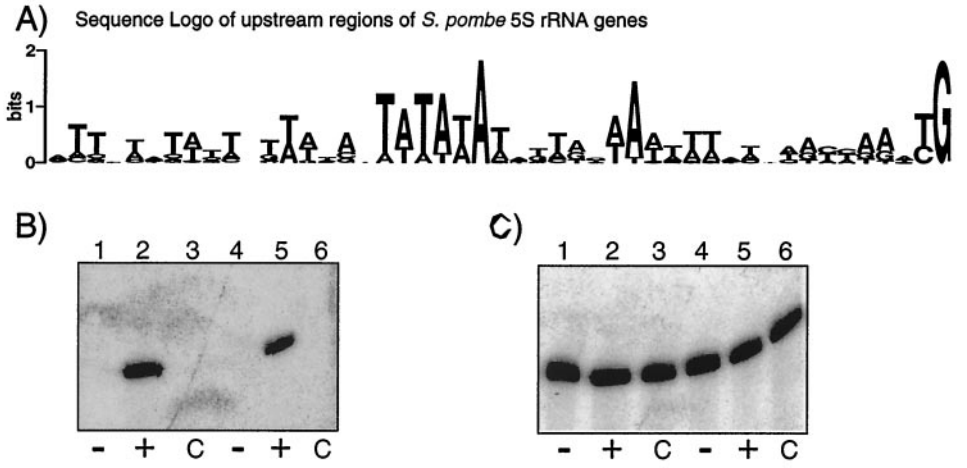

FIG. 3. Essential TATA motifs upstream of *S. pombe* 5S rRNA genes. (A) Sequence Logo of the upstream regions of multiple dispersed *S. pombe* 5S rDNA loci. The first base (G) of mature 5S rRNA was included as the last base on the right. (B) Northern blot analysis. A neutral sequence tag allows detection of the tagged transcript, designated 5S* rRNA, which is distinguishable from endogenous 5S rRNA. In vivo expression of plasmid-borne TATA-containing (+) and TATA-less (−) 5S* rRNA genes was monitored with a 5S*-specific probe. C, control. (C) The blot in panel B was stripped and rehybridized to detect endogenous 5S rRNA.

mid, and the transformants exhibited indistinguishable growth phenotypes (not shown). Total RNAs from duplicate sets of transformants were examined by Northern blotting (Fig. 3B). Hybridization with an antisense oligonucleotide probe revealed that 5S rRNA was expressed from the 5S* gene containing the wild-type TATA (Fig. 3B, lanes 2 and 5) but not from the TATA-less 5S* gene (lane 1 and 4) or from the control plasmid lacking an insert (lanes 3 and 6). To estimate the relative activity of the TATA-containing 5S* gene and to examine for differences in loading, the blot was stripped and rehybridized with an oligonucleotide specific for the wild-type 5S rRNA sequence under the same conditions as for the 5S* probe (Fig. 3C). Quantitation led to the estimate that 20 to 25% of the total 5S rRNA in the TATA-containing lanes was produced by the plasmid-borne 5S* rRNA gene containing the wild-type TATA element, while the TATA-less gene produced background levels of 5S* rRNA (not shown). These results indicated that the conserved TATA found upstream of *S. pombe* 5S rRNA genes is required for 5S rRNA expression in vivo. Sequence analysis revealed that these constructs differed only in the upstream TATA element, as expected (not shown). Therefore, the large difference in the amount of 5S* rRNA production from the TATA-containing and TATA-less 5S* rRNA genes suggests that TATA-dependent transcription represents an obligatory pathway of Pol III recruitment in *S. pombe* cells.

The Brf subunit of TFIIIB copurifies with fission yeast TFIIIC and Pol III with no associated TBP. Extracts of a previously described *S. pombe* strain carrying FLAG-tagged Sfc3p, the *S. pombe* homolog of the B box-binding subunit of TFIIIC (21); a new strain carrying FLAG-tagged spRet1p, the *S. pombe* homolog of the second-largest subunit of Pol III; and a control strain, yAS50, were subjected to immunoaffinity purification using monoclonal anti-FLAG antibody (M2) cross-linked to agarose (Fig. 4). The input, flowthrough, and affinity-eluted materials were subjected to immunoblotting to detect the proteins indicated to the right of each panel. The spBrf homolog was found associated with Sfc1p, Sfc4p, Sfc6p, and the FLAG-tagged Sfc3p (Fig. 4C to F and H, lanes 6), while two other TFIIIB homologs, spTBP and spB″, were not associated (Fig. 4G and I, lanes 6). The relative amounts of Brf in the negative control (lanes 3) and the Sfc3p-TFIIIC complex (lanes 6) are comparable to the ratio seen for Sfc6p, a genuine TFIIIC component (lanes 3 and 6). Quantitative analysis using known amounts of recombinant proteins indicated that a substantial fraction of spTFIIIC is specifically associated with Brf (not shown). The Pol III subunit homologs, spRet1p and spRpc39p were not associated with the *S. pombe* TFIIIC complex (Fig. 4A and B, lanes 6), nor was the Pol III nascent transcript-binding protein, spLa (Fig. 4J, lane 6), further indicating specificity of the Brf association. The same profile was also reproducible when a strain carrying epitope-tagged Sfc6p was used for purification (not shown). These results indicate that in *S. pombe*, Brf is specifically associated with a substantial fraction of TFIIIC, and most significantly, this occurs in the absence of TBP.

Analysis of the spRet1p complex revealed the presence of the associated Pol III subunit, spRpc39p, as expected (Fig. 4A and B, lanes 9). In this case, spBrf and spB″ homologs were reproducibly found associated with the complex, while spTBP, spLa, and four spTFIIIC subunits were not associated (Fig. 4C to J, lanes 9). The specificity of the Brf association with the TFIIIC and Pol III complexes was reproducible in multiple experiments and was observed with affinity-purified antibody raised against a terminal peptide of spBrf (Fig. 4), as well as with two antisera raised against recombinant spBrf (not shown). The same profile of associated and nonassociated proteins was observed when a strain carrying the spRPC53p subunit of Pol III was epitope tagged and used for purification (not shown; as will be described elsewhere, FLAG-tagged Pol III is active for promoter-dependent transcription, as well as efficient termination and recycling on preassembled transcription complexes isolated on immobilized template DNA).

It was noted that TFIIIB components could not be detected

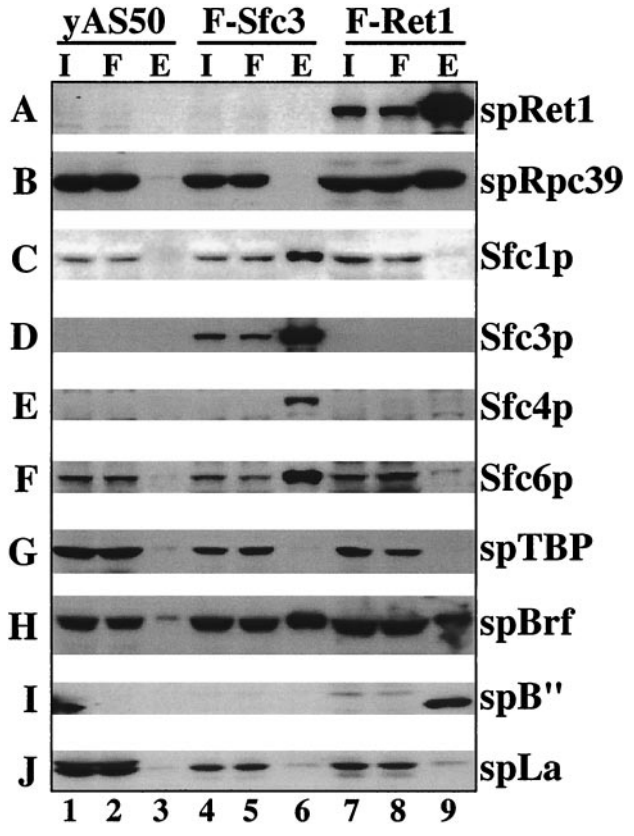

FIG. 4. Fission yeast Brf and B″ associate with Pol III TF complexes in the absence of stably associated TBP. Extracts prepared from a wild-type strain, the FLAG-*Sfc3* strain, and a FLAG-spRet1p strain were incubated with anti-FLAG immunoglobulin G (M2)-agarose. After incubation, the supernatants were collected as the flowthrough, the agarose was washed five times with buffer containing 250 mM NaCl, and the bound material was eluted. The input (I), flowthrough (F), and eluate (E) of the M2-agarose from the three extracts were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by immunoblotting using antisera to the proteins as indicated on the right.

when the epitope-tagged TFIIIC220 human counterpart of Sfc3p was used to isolate TFIIIC complexes from HeLa cells (74), suggesting that the Brf-TFIIIC association may be a unique characteristic of *S. pombe*. TBP together with Brf was readily found associated with epitope-tagged Pol III isolated from *S. cerevisiae* (7). spTBP could not be detected in our Brf-containing spTFIIIC complex or the Brf-containing Pol III complex, even though TBP is found tightly associated with Brf (in the absence of DNA) in other systems (20, 22, 31, 45, 55, 75). Thus, compared to the TFIIIB subunit associations in other systems, the *S. pombe* TFIIIB subunits appear to be arranged differently, although in a manner that is appropriate for the unique promoter architecture of *S. pombe* tRNA genes (see below). Furthermore, we have been unable to demonstrate association between TBP and Brf in *S. pombe* extracts by using direct immunoprecipitation or epitope-tagging approaches (Y. Huang, M. Weindel, and R. Maraia, unpublished data). The apparent weak association of TBP and Brf would appear to reflect a functional feature of the *S. pombe* Pol III system: principally TATA-mediated, rather than principally Brf-mediated, recruitment of TBP to the core promoter regions of tRNA genes.

Requirement for an upstream TATA element to program a functional Pol III preinitiation complex. Since tRNA expression in *S. pombe* involves the widespread use of upstream TATA elements, we wanted to examine another hallmark feature of class III genes, their ability to program stable transcription complexes (15). While the interaction of TFIIIC with certain tRNA (and VA1) genes generates a complex that is stable on subsequent challenge with orthologous promoter DNA, this is not true for other tRNA genes, in large part due to variances in the A and/or B box elements (1, 12, 29, 37). However, inclusion of TFIIIB in the TFIIIC-DNA complex does program stable complex formation (33, 37). According to the assembly pathway elucidated using *S. cerevisiae* components, TFIIIC binds to the internal promoter and then recruits TFIIIB to the upstream DNA through contacts made principally to Brf (6, 57, 60). Thus, on TATA-less promoters, TFIIIC recruits and induces Brf and associated TBP to bind upstream DNA (6, 29, 47, 57, 60; reviewed in reference 7). This assembly pathway leads to a preinitiation complex that is stable upon challenge by homologous promoters and is consistent with the assemblages elucidated for human, *Drosophila*, and *Xenopus* (3, 30, 37, 59, 76). Using currently available activities, we can examine complex assembly and stability with a template commitment assay (3, 37, 61). In this assay, the first template is allowed to assemble with TFs and a second template is then added to challenge the stability of the first transcription complex. Because several templates were compared in *S. cerevisiae* and *S. pombe* extracts (Fig. 5), multiple observations regarding *cis*-acting elements and *trans*-acting factors are noteworthy.

Three tRNA gene templates, in TATA-containing and TATA-less versions, were examined simultaneously in *S. cerevisiae* and *S. pombe* extracts (Fig. 5, top and bottom, respectively). The *S. cerevisiae* $tRNA^{ser}$ gene contains A and B elements that match the consensus, while the A and B box elements of the *S. pombe* $tRNA^{ser}$ genes differ from those of the *S. cerevisiae* gene at one position only, T at position 10. Both the TATA-containing and TATA-less versions of the *S. cerevisiae* $tRNA^{ser}$ gene (Fig. 5, templates A and B) were efficiently transcribed and excluded transcription of the challenging tRNA gene (template F) in *S. cerevisiae* extract (top, lanes 1 and 2). In contrast to this, while either of the *S. cerevisiae* tRNA genes could exclude the second template in *S. pombe* extract, only the TATA-containing gene (template B) was active for transcription while the TATA-less gene (template A) was inactive (bottom, lane 1 and 2). Most significantly, this indicates that although the tRNA gene is able to interact stably with limiting *S. pombe* TFs, this alone is not sufficient to program a functional transcription complex in the absence of a TATA element.

Both versions of the *S. pombe* $tRNA^{ser_{UGA}}$ gene excluded transcription from the second template in *S. cerevisiae* extract (Fig. 5, top, lanes 3 and 4), although the TATA-containing gene (template D) was transcribed more efficiently than the TATA-less gene (template C). Prior data suggest that the relatively low-level transcription of the *S. pombe* gene in *S. cerevisiae* extract is almost certainly due to the single nonconsensus nucleotide, T10 (48) (Fig. 5, top, compare lanes 1 and 3).

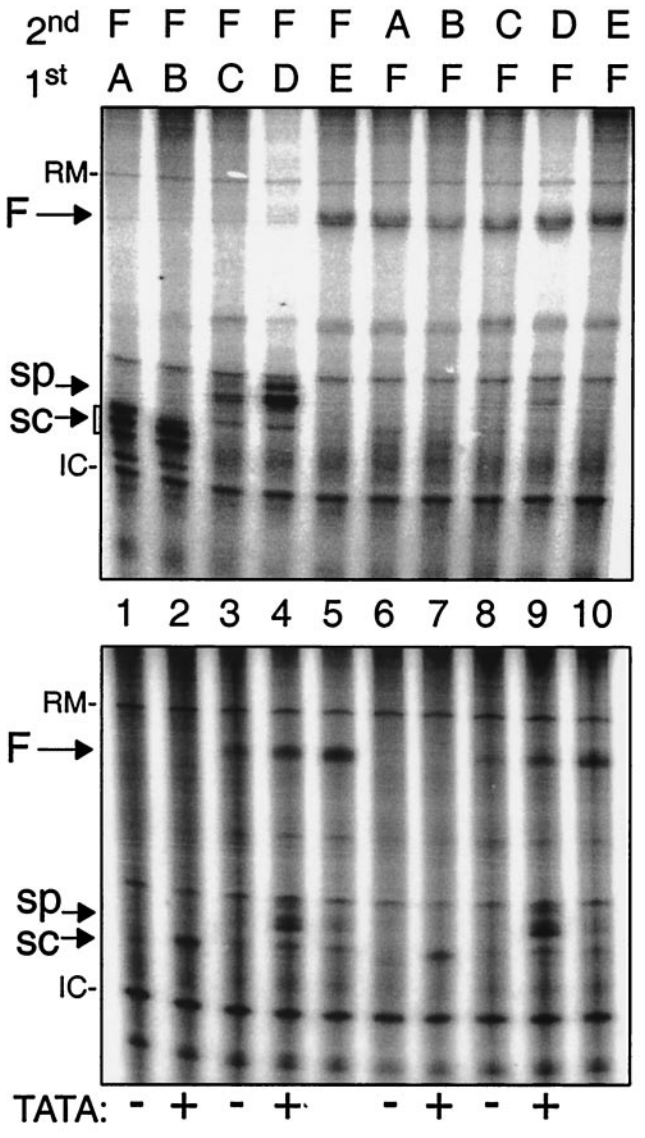

FIG. 5. An upstream TATA is required to program a functional Pol III preinitiation complex on an A box- and B box-containing tRNA gene in *S. pombe*. Transcription complexes were monitored using template exclusion assays performed in parallel in *S. cerevisiae* (top) and *S. pombe* (bottom) in vitro transcription systems. The tRNA genes used for Fig. 2B were tested for the ability to form stable transcription complexes. After incubation of the first template (the order of addition is indicated above the lanes) with transcription buffer (previously determined to be optimal at 20 min), nucleoside triphosphates and [α-^{32}P]GTP were added along with the second template (indicated above the lanes), and transcription was allowed to proceed. The reaction was stopped, and RNA was extracted and separated on 6% polyacrylamide–8 M urea. The templates were as follows: A, sc-tRNA^{Ser} −TATA; B, sc-tRNA^{Ser} +TATA; C, sp-tRNA^{Ser} −TATA; D, sp-tRNA^{Ser} +TATA; E, pJK148 (empty vector); F, sp-tRNA^{Ser}-3T +TATA. The arrows point to the different transcripts synthesized.

In contrast to the stability observed in the *S. cerevisiae* system with the *S. pombe* tRNA$^{ser_{UGA}}$ gene, this gene did not exclude transcription from the second gene (template F) in the *S. pombe* system (Fig. 5, lanes 3 and 4). Because the promoters of the *S. cerevisiae* and *S. pombe* genes differ at only one position in the internal promoter (G10 versus T10, respectively), which is a significant determinant of transcription (48), and because prior studies indicate that interaction of TFIIIC with the A box can be a determinant of template exclusion (1, 37, 61, 65), it is reasonable to deduce from the data in Fig. 5 that *S. pombe* TFIIIC stably interacts with the consensus promoter (templates A and B, lanes 1 and 2) but not with the nonconsensus promoter (templates C and D, lanes 3 and 4) and that this is the basis of template exclusion in the former but not the latter. Moreover, Fig. 5 provides additional information that is relevant to a major conclusion of this study: even in the case where a stable complex is formed in the *S. pombe* system (Fig. 5, bottom, lanes 1 and 2), presumably reflecting a stable interaction of TFIIIC with the consensus promoter (37), this is not sufficient to program a functional preinitiation complex in the absence of a TATA element. By contrast, stable TFIIIC binding is sufficient to recruit functional TFIIIB in the *S. cerevisiae* system (29, 30, 33, 61, 65) (Fig. 5, top). The cumulative data in Fig. 5 argue strongly that a TATA element is required for the functional programming of an *S. pombe* transcription complex even when all of the necessary activities are present.

The results support the model in which the TFIIIC-mediated placement of TFIIIB, which leads to a functional transcription complex in *S. cerevisiae*, does not appear to occur efficiently, if at all, in the *S. pombe* system (Fig. 5, bottom, lanes 1 and 2). A second point that is suggested by the data is that the *trans*-acting factors that recognize TATA to promote transcription need not be recruited into a highly stable complex in the *S. pombe* system, since even when a functional complex is assembled, it is not stable on challenge by the orthologous promoter (Fig. 5, bottom, lane 4). This unexpected observation suggests that *S. pombe* TFIIIB interacts less stably with tRNA transcription complexes than does *S. cerevisiae* TFIIIB (see Discussion). The demonstrable differences in the two transcription systems were confirmed and strengthened by results obtained with control templates and were also observed when the order of addition of the genes was switched (lanes 5 to 10). The results provide convincing biochemical evidence that the *S. pombe* and *S. cerevisiae* transcription systems interact differently with tRNA genes but in a manner that is consistent with the associations of TFIIIB subunits shown in Fig. 4.

A TATA element in the *S. pombe* Pol I core promoter. TATAAAA was also found in the region upstream of the transcription initiation sites of the genes that encode large rRNAs. In this promoter, TATAAAA overlaps the −30 region of the previously determined Pol I start site (Fig. 6A) (8). We examined 5.8S rRNA expression from a plasmid that harbors the TATA-containing wild-type 37S rRNA gene of *S. pombe*. The 5.8S rRNA produced from this plasmid is marked with a 4-nucleotide sequence tag that allows its transcript (designated 5.8S*) to be distinguished from endogenous 5.8S rRNA by slower mobility on polyacrylamide gels (23). The upstream TATAAA was changed to GGATCC (Fig. 6A) or left unaltered, and the resulting plasmids were used to transform ura4-D18 *S. pombe* cells to the *ura4*$^+$ phenotype. The two plasmids yielded comparable transformation efficiencies, and the transformants exhibited indistinguishable growth phenotypes (not shown). Total RNA was prepared from the transformants and examined by polyacrylamide gel electrophoresis and ethidium bromide staining (Fig. 6B). As described, the tagged 5.8S*

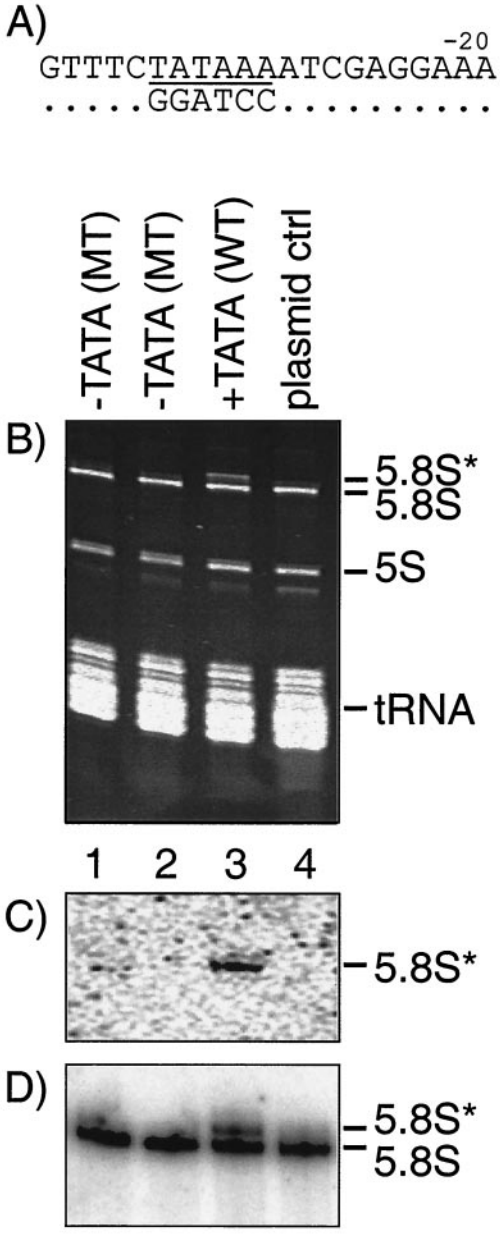

FIG. 6. Pol I-transcribed large-rRNA genes use an upstream TATA motif in the core promoter region. (A) Sequence of promoter region (−40 through −20 relative to +1), with TATAAA motif underlined, of *S. pombe* rDNA (top line) (8) and TATA mutant (bottom line); dots indicate identical residues. (B) Ethidium bromide-stained polyacrylamide gel. Total RNA was isolated from *S. pombe* strains that had been transformed with plasmids containing 37S rRNA genes in which the 5.8S rRNA sequence contained a unique sequence tag (5.8S*; see the text). Lanes 1 and 2, two independent isolates in which the TATA element was mutated by site-directed substitution; lane 3, wild-type TATA; lane 4, control plasmid containing no rRNA gene. (C) Northern blot analysis using oligonucleotide probe conditions specific for the tagged 5.8* rRNA; the lanes are the same as in panel B. (D) The Northern blot in panel B was stripped and hybridized using an oligonucleotide probe that recognizes both wild-type (endogenous) 5.8S rRNA and 5.8* rRNA; the lanes are the same as in panel B.

rRNA was visible as a clear band with slower mobility than 5.8S rRNA (23), produced from the wild-type TATA-containing plasmid (lane 3). Cells containing plasmids bearing the TATA-less promoter (lanes 1 and 2) and the control plasmid lacking the rRNA gene (lane 4) did not express the 5.8S* rRNA. As a control, the TATA-less promoter was converted back to a TATA-containing promoter by site-directed mutagenesis, and this rescued 5.8S* rRNA (not shown). Hybridization with an antisense probe confirmed that the tagged 5.8S* rRNA was expressed from the gene containing the wild-type TATA (Fig. 6C, lane 3) but not from the TATA-less genes or the control plasmid (lanes 1, 2, and 4). An oligonucleotide complementary to wild-type 5.8S and 5.8S* rRNAs revealed comparable loading in all lanes (Fig. 6D); again as expected, 5.8S* was expressed only from the TATA-containing plasmid (Fig. 6D, lane 3). These results indicated that the TATAAA sequence that is naturally found upstream of large rRNA genes in *S. pombe* is important for rRNA expression in vivo.

DISCUSSION

A major conclusion of this study is that transcription in *S. pombe* involves widespread use of TATA promoter elements. This is significant not so much because of the number of promoters involved but more so because TATA use is widespread in the Pol I and III systems. It has generally been found that, except for rare exceptions (see below), eukaryotic tRNA gene promoters are TATA-less. The results presented here should extend the known diversity of gene structure and promoter function in eukaryotes (59, 66). Although, as detailed below, scattered reports indicate TATA elements upstream of some tRNA and 5S rRNA genes, this is the first report that indicates TATA function for the great majority of tRNA, 5S rRNA, and large-rRNA genes. Moreover, for these genes, TATA-mediated transcription is an obligatory pathway of expression, indicating that functional use of TATA elements is the rule for tRNA transcription in *S. pombe* rather than the exception (see below). The inability to detect expression from TATA-less tRNA and 5S rRNA genes in *S. pombe* provides strong evidence to suggest that the mechanism to bring TFIIIB activity to the core promoter in the absence of a TATA element that has been elucidated for other model organisms is not available to and/or not used in *S. pombe*. This further suggests a fundamental difference between fission yeast and other model systems in the mechanisms that bring the central TF, TBP, to the core promoter. The presence of TATA elements upstream of a substantial fraction of *A. thaliana* tRNA genes suggests that plants and perhaps other branches of the evolutionary tree share this feature with fission yeast. Thus, this study provides a basis to suggest that, contrary to what has been generally accepted, a significant portion of eukaryotes may use TATA-dependent mechanisms for the great majority of their Pol III transcription.

***S. pombe* Pol III promoters: TATA elements are the rule.** As derived from Fig. 1, the consensus sequences 8TRG/NYNNA RNNG18 and 53RTTCRANYY62 represent the A and B boxes of *S. pombe* tRNA genes. For the *S. pombe* 7SL and U6 RNA genes, sequence matches to the A box begin 13 and 17 bp, respectively, downstream of their first nucleotide. These genes also match the B box consensus sequence beginning at

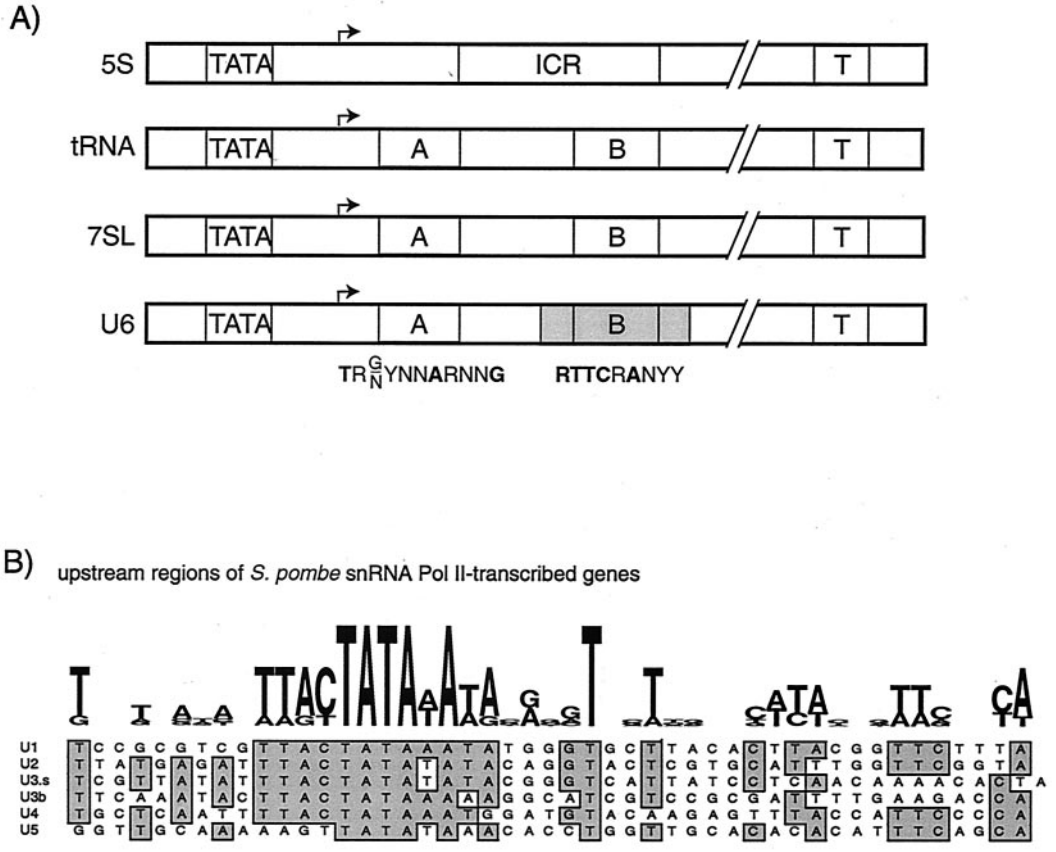

FIG. 7. Conserved TATA motifs in the promoters of small RNA genes in *S. pombe*. (A) Pol III-transcribed genes. The upstream TATA, internal control region (ICR) (for 5S only), A box and B box (for tRNAs and U6 and 7SL RNAs), and terminators (T) are shown. The bent arrows indicate the +1 positions (as previously determined). Consensus sequences for the A and B box elements that fit all tRNA genes, as well as U6 and 7SL genes, are shown below the schematics; boldface letters are invariant or very highly conserved. The U6 B box resides in an intron (shaded; see the text) (B) Upstream regions of Pol II-transcribed snRNA genes are presented both in Logo (above) and as individual aligned sequences, ending at +1.

positions 60 and 69 of 7SL and U6, respectively. Substitution of conserved B box residues in the *S. pombe* U6 gene rendered this template inactive in the *S. pombe* in vitro transcription system (M. Hamada and R. Maraia, unpublished data). Thus, in *S. pombe*, tRNA, U6, and 7SL genes exhibit similar promoter architectures, in which TATA appears as one of multiple promoter elements (Fig. 7A).

The data in Fig. 5 suggest that although tRNA genes with A box residues other than G at the number 10 position represent a significant fraction of genes, these may interact with TFIIIC in a less stable manner than those with G at this position. Attempts to identify other features of these genes that distinguished them from the main set of tRNA genes was not successful. It is also interesting in this regard that the A box sequences of the *S. pombe* U6 and 7SL genes contain a G at the corresponding position of their A box sequences. In considering the potential implications of these findings, it may be important to recall that both the G10 and the non-G10 tRNA genes required an upstream TATA for expression in our assays. While the results suggest a potential for effects of the A box residue corresponding to G10 related to transcription complex formation and/or stability, the physiological significance of this, if any, remains an open question.

Species specificity of Pol III-dependent tRNA transcription. It is important to note that the data indicate that the role of TATA is not to provide simple additive transcriptional activity to the internal promoters of tRNA and 5S rRNA genes in *S. pombe*; rather, the homologous transcription machinery is highly dependent on and functionally adapted to the TATA element, even when the internal promoter is strong (Fig. 2, bottom, lanes 1 and 2).

The requirement for an upstream TATA may explain, at least in part, observations that indicate differential expression of tRNA genes in the two yeasts such that while *S. pombe* tRNA genes are efficiently expressed in *S. cerevisiae*, the TATA-less tRNA genes from *S. cerevisiae* were not expressed in *S. pombe* (34, 79).

Intriguingly, the data in Fig. 1 reveal an inverse correlation between the prevalence of guanosine at position 10 and the presence of an upstream TATA motif, most readily observable for *S. cerevisiae*, *S. pombe*, and *A. thaliana*. Since G10 is invariant or highly conserved in species that rely on TATA-less transcription, the data suggest that G10 may be a significant determinant of TATA-less transcription. This is consistent with the data in Fig. 5 (top). For templates bearing T10 but no TATA, transcription activity is low relative to the same tem-

plate with TATA (compare lanes 3 and 4), while for templates containing G10, the presence or absence of TATA makes little if any difference (compare lanes 1 and 2).

TATA elements have been noted upstream of tRNA genes in *A. thaliana*, and a TATA element has been reported to effect reinitiation of tRNA transcription in tobacco (13, 81). A CAA motif was noted to be a transcription initiation site for plant tRNA (81). The analysis in Fig. 1B suggests that this motif may be extended to ANCAANA/T (the underlining reflects a +1 site), reminiscent of initiator elements (Inr) found in some Pol I and Pol II genes (56, 66). However, several differences distinguish the patterns of sequence information content in *S. pombe* and *A. thaliana* tRNA genes (Fig. 1B): (i) the information content is higher at TATA for *S. pombe*, (ii) the information content is higher at position 10 (greater propensity for G10) in *A. thaliana*, and (iii) the information content is higher around +1 for *A. thaliana*. The *A. thaliana* pattern is consistent with data that show partial or little effect of TATA mutations but larger effects of CAA mutations on plant tRNA expression (9, 81). Thus, the cumulative evidence indicates that although fission yeasts and plants exhibit TATA elements upstream of their tRNA genes, *S. pombe* is more obligatorily dependent on the TATA promoter element for transcription.

TATA and tRNA transcription: exceptional cases in conventional model organisms. Although in certain cases TATA elements direct Pol III transcription of tRNAs, these appear to be unusual instances. The transcription start site for the gene encoding *Xenopus laevis* selenocysteine $tRNA^{[ser]sec}$ is dictated by an upstream promoter that includes a TATA element (5, 39). However, this gene utilizes a unique pathway of expression, as evidenced by the Pol III initiation site, which occurs at a highly unusual position for a tRNA, coinciding with the 5′ end of the mature tRNA (38). The sequence of $tRNA^{[ser]sec}$ does not contain an A box promoter element, and start site selection is instead mediated by the TATA element (5, 52). An upstream TATA has also been shown to control expression of a tRNA gene in the insect *Bombix mori*, in which two genes contribute to silk gland-specific $tRNA^{Ala}$ synthesis in a tissue-regulated manner. In this case, an upstream TATA directs expression of the more active of the two $tRNA^{Ala}$ genes in a TBP-dependent manner (50). This $tRNA^{Ala}$ gene was also analyzed in *D. melanogaster* cells, where its transcription was stimulated by increased TBP levels (71). Figure 1A demonstrates that TATA elements are not found upstream of most tRNA genes in the insect *D. melanogaster*, for which it was recently reported that TBP-related factor, TRF, rather than TBP itself, directs tRNA transcription (68). The cumulative data are consistent with a model in which TATA and TBP may be used for special cases of tRNA expression in insects and other organisms. In summary, prior to the present report, TATA elements had been characterized as promoters of tRNA transcription in exceptional cases.

A functional TATA element in a Pol I core promoter. Our data demonstrate that substitution of the TATA element that overlaps the −30 region of the *S. pombe* Pol I promoter abolishes rRNA expression in vivo. Although our results do not address the mechanism by which TATA promotes expression by Pol I in *S. pombe*, they nonetheless indicate an important role of TATA in rRNA expression in fission yeasts. The location of the TATAAA sequence, positioned 35 bp upstream of the previously determined start site of Pol I transcription, suggests that TATA-TBP functions as part of the core promoter in *S. pombe*.

TATA elements are also more widespread in the Pol II-transcribed genes in *S. pombe* than in other species. Although TATA promoter elements are a recognized hallmark of protein-encoding genes, not all Pol II promoters contain TATA elements, as some mRNA-encoding genes use other core promoter elements for transcription (4, 66). Another class of genes, those that produce snRNAs (e.g., U1 to U5), are transcribed by Pol II from TATA-less promoters in yeast, human, and *Drosophila* (18, 73, 82). In contrast to this, TATA motifs have been noted in the promoter regions of some *S. pombe* snRNA genes that are transcribed by Pol II (reference 83 and references therein). Alignment of the upstream regions of the U1 to U5 snRNA genes of *S. pombe* revealed a prominent TATA motif in addition to an upstream TTAC sequence (Fig. 7B).

Evolutionary implications. It seems reasonable to consider *S. pombe* a tentative link to an ancient ancestor that used ubiquitous TATA elements to promote transcription of a wide range of genes, if not of all genes. The ubiquitous TATA-like elements of an archaeonlike organism would have remained after divergence of the three nuclear Pols. Thus, while *S. pombe* may have retained TATA elements as essential components of the Pol I, II, and III systems, many genes of other organisms would have lost their TATAs. This scenario is consistent with a residual requirement for TBP and/or TBP-related factors by all three Pols, even though most of their target genes do not use TATA elements in many species. It is also consistent with the use of TBP-related factors in metazoans (19, 68, 69, 72). Although plant tRNA genes are also flanked by upstream TATAs (13) (Fig. 1), we are unaware of a simple line of inheritance between plants and *S. pombe* that could account for this common feature. 5S rRNA genes are also dispersed in *Neurospora crassa*, and transcription was also reported to require a TATA at −29 (64). TATA elements upstream of a substantial fraction of plant and other species' 5S and tRNA genes suggests that fission yeasts are not unique in the structure of their Pol III promoters.

The transcription system characterized here suggests that Pol III initiation in *S. pombe* may be comparable to type 3 gene transcription and other systems that use TATA elements to recruit TBP. Although for tRNA transcription, the yeast and mammalian TFIIIB subunits appear to be completely orthologous, the situation is more complex for U6 snRNA expression in metazoa (e.g., type 3 genes), as a distinct variant of BRF known as BRFU/TFIIIB50 characterizes the TFIIIB activity that mediates type 3 gene transcription (63, 70). The BRFU/TFIIIB50 variant that acts at the U6 TATA-containing promoter differs from Brf in its lack of a C-terminal domain that in Brf has been shown to interact with TBP (10, 26, 32), and consistent with this, it may not stably bind TBP, since association of TFIIIB with TBP was notably weak (70). This suggests principal recruitment of TBP by the TATA element, which may obfuscate a requirement for stable interaction between TBP and BRFU in the absence of DNA (70). In this regard, the *S. pombe* Pol III initiation system may be comparable to Pol II, in which TBP and TFIIB do not form a stable association in the absence of DNA. Instead, association of TBP with

the TATA element appears to create a binding site for TFIIB, in part due to the specific bending effect of TBP on TATA DNA (49). While our results indicate a significant role for TATA in tRNA transcription in *S. pombe*, they also leave open the possibility that Brf-mediated recruitment of TBP occurs at some level in vivo and that some tRNA genes are less dependent on the TATA element for TBP recruitment than others. However, the cumulative data suggest that TATA-mediated recruitment of TBP plays a major role in Pol III transcription in *S. pombe*, while Brf may be brought to the upstream region in part by TFIIIC and in part by recognition of the TATA-TBP complex. In any case, *S. pombe* has revealed a novel Pol III system that should be useful for studying eukaryotic transcription initiation and the function of the core promoter.

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Mitsuhiro Hamada and Ying Huang contributed equally to this work.

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