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Architecture of a Yeast U6 RNA Gene Promoter

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The promoters of vertebrate and yeast U6 small nuclear RNA genes are structurally dissimilar, although both are recognized by RNA polymerase III. Vertebrate U6 RNA genes have exclusively upstream promoters, while the U6 RNA gene from the yeast *Saccharomyces cerevisiae* (*SNR6*) has internal and downstream promoter elements that match the tRNA gene intragenic A- and B-block elements, respectively. Substitution of the *SNR6* A or B block greatly diminished U6 RNA accumulation in vivo, and a subcellular extract competent for RNA polymerase III transcription generated nearly identical DNase I protection patterns over the *SNR6* downstream B block and a tRNA gene intragenic B block. We conclude that the *SNR6* promoter is functionally similar to tRNA gene promoters, although the effects of extragenic deletion mutations suggest that the downstream location of the *SNR6* B block imposes unique positional constraints on its function. Both vertebrate and yeast U6 RNA genes have an upstream TATA box element not normally found in tRNA genes. Substitution of the *SNR6* TATA box altered the site of transcription initiation in vivo, while substitution of sequences further upstream had no effect on *SNR6* transcription. We present a model for the *SNR6* transcription complex that explains these results in terms of their effects on the binding of transcription initiation factor TFIIIB.

Nuclear RNA polymerases recognize their transcription units by binding to accessory initiation factors stably complexed with short promoter elements. Genes transcribed by RNA polymerase III (RNAPIII), which synthesizes many small RNAs, typically have intragenic promoter elements. tRNA genes, for example, contain two such elements, called the A and B blocks (22). These two 11- to 12-bp DNA sequences are binding sites for transcription factor IIIC (TFIIIC), which in turn positions another factor, TFIIIB, immediately upstream of the tRNA gene (28). TFIIIB then directs initiation by RNAPIII (28). Transcription of a silkworm tRNA gene in a homologous cell extract requires an additional factor composed of RNA, called TFIIIR (60). It is not known whether TFIIIR is required for tRNA gene transcription in other organisms.

U6 RNA, the smallest of five small nuclear RNAs found in the spliceosome, is synthesized by RNAPIII (32, 47), while the other four spliceosomal small nuclear RNAs are made by RNAPII. In contrast to tRNA genes, vertebrate U6 RNA genes have no intragenic promoter elements. Instead, their promoters lie entirely upstream and include a TATA box-like sequence at position −30 and a proximal sequence element (PSE) between positions −70 and −50 (11, 15, 33, 34, 37, 44). Such upstream RNAPIII promoters (reviewed in reference 31) have been termed type 3 to distinguish them from the type 2 promoter of tRNA genes and type 1 promoter of 5S rRNA genes (23). It is not known how RNAPIII is able to recognize type 3 promoters (14). The key role of TFIIIB in the recruitment of RNAPIII for tRNA and 5S rRNA gene transcription, and the finding that a TFIIIB-containing fraction is required for vertebrate U6 gene transcription (56), suggest that the U6 gene preinitiation complex contains TFIIIB bound immediately upstream of the start site. However, the protein-protein and protein-DNA interactions responsible for the presumptive positioning of TFIIIB upstream of vertebrate U6 RNA genes are unknown. The absence of A- and B-block promoter elements argues against the involvement of TFIIIC. The surprising discovery that in vitro transcription of vertebrate U6 genes requires the TATA-binding protein (TBP) subunit of RNAPII initiation factor TFIID (38, 51) and an RNAPII factor that stabilizes TBP binding to the TATA box, TFIIA (55), raises the possibility that TFIIIB is positioned by TBP and associated proteins bound to the vertebrate U6 gene TATA box.

We have previously isolated the U6 RNA gene, *SNR6*, from the yeast *Saccharomyces cerevisiae* and found that unlike vertebrate U6 genes, it contains an essential B-block promoter element (7). The *SNR6* B block is in a unique position, downstream of the coding region (see Fig. 1). A candidate A-block element in the conventional intragenic position was also identified, and it was suggested that the yeast U6 RNA gene binds TFIIIB by the same TFIIIC-dependent mechanism used by tRNA genes (7). However, the yeast U6 gene has upstream sequences similar to the vertebrate U6 gene TATA box and PSE (6), and Sentenac and coworkers have shown that an allele of *SNR6* that lacks the downstream B block can be transcribed in vitro in a fractionated transcription system (46). Furthermore, under such conditions, yeast U6 gene transcription does not require TFIIIC but does require TBP (43). These findings led to the suggestion that the basal promoter of the yeast U6 gene lies completely upstream, as it does in vertebrate U6 genes, and that the downstream B block acts as an enhancer of transcription (21).

Here we report that most of the sequence upstream of the yeast U6 gene is dispensable for transcription in vitro and in vivo, while an intragenic A block and the downstream B block are essential for expression. DNase I footprinting confirms that the factor that binds to the downstream B block has properties expected of TFIIIC. Our results support the hypothesis that *SNR6* transcription requires binding of TFIIIC to the A- and B-block elements and indicate that the yeast U6 RNA gene promoter is more similar to type 2

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than to type 3 RNAPIII promoters. Nevertheless, we found that the *SNR6* TATA box is required for accurate initiation, consistent with a role for TBP in initiation complex formation. Furthermore, unexpected effects of extragenic deletions on *SNR6* promoter strength suggest that the unique downstream location of the yeast U6 RNA gene B block imposes special constraints on the spacing of promoter elements.

MATERIALS AND METHODS

Plasmid constructions. The yeast 5S rDNA clone pB-1 was described previously (5). The yeast $tDNA_3^{Leu}$ clone pPC1 is the 341-bp *Rsa*I-*Msp*I fragment of YEp13 cloned into the *Hin*cII site of pUC12 and was kindly provided by E. P. Geiduschek. *SNR6* clones pEP6, p-539H6, pCH6, pCH6ΔB, pCS6, pCD6, and pCCs6 have been described previously (7). The *SNR6* deletion and substitution constructs described in this report were derived from pEP6, p-539H6, pCCs6, or $pY3'H_{SB}$ (kindly provided by Rémy Bordonné [3]). Briefly, pEP6 contains the yeast genomic sequence from −539 to +830 (relative to the transcription start site of *SNR6*), p-539H6 contains the yeast sequence from −539 to +630, pCCs6 contains the yeast sequence from −120 to +235 (and lacks most of the essential B-block promoter element, from +234 to +244), and $pY3'H_{SB}$ is a chimeric construct of *SNR6* (yeast sequence from −539 to +630) with the human U6 sequence in the 3′ half of the U6 coding region. p-539H6, pEP6, and pCCs6 are contained in vector pUC118 (54), while $pY3'H_{SB}$ is contained in vector pBluescript (Stratagene).

pΔBE6 (Δ59-72) was generated by digestion of pEP6 with *Bcl*I and *Eco*NI, filling in with the Klenow fragment of DNA polymerase (New England Biolabs), and ligation with T4 DNA ligase (U.S. Biochemicals). pΔNB6 (Δ6-54) was generated by digestion of pEP6 with *Nru*I and *Bcl*I, filling in with Klenow fragment, and religation. pΔBBg16 (Δ65-108) was generated by digestion of $pY3'H_{SB}$ with *Bcl*I and *Bgl*II, filling in with Klenow fragment, and religation. This deletion resulted in removal of all but 6 bp of the human U6 sequence from the parental yeast-human chimeric gene. Thus, the construct is a deletion of bp 65 to 108 of the yeast U6 sequence and a substitution of the sequence AGCAGT (positions 59 to 64 of *SNR6*) with the sequence TCCATA. p-39H6 was generated by digestion of pPSE-sub (see below) with *Kpn*I and treatment with S1 nuclease (U.S. Biochemicals) at 37°C for 30 min. The S1 nuclease cleaved internally in A-T-rich regions, so subsequent cleavage with *Eco*RI, filling in with Klenow fragment, and religation resulted in upstream deletions reaching beyond the *Kpn*I site generated by the PSE substitution. p-39D6 was made by digestion of p-39H6 with *Dde*I, filling in with Klenow fragment, and digestion with *Eco*RI. The *Eco*RI-*Dde*I fragment was then ligated into *Eco*RI-*Hin*cII-cut pUC118. Plasmids pΔAB6 (Δ32-53), pA-sub (A-block substitution), pΔ138-221, pΔ138-179, pΔ180-221, pIgBB+B (intragenic B-block mutation), pPSE-sub, pIn-sub, pTATA-sub, pAATA-sub, pA1-sub, and pA2-sub were generated by oligonucleotide-directed mutagenesis of p-539H6 (or pTATAbox-sub for double mutants). pIgBB was made by mutagenesis of pCCs6. pTATAbox-sub was made by mutagenesis of pTATA-sub. Plasmids p-539H6, pCCs6, pTATA-sub, and pTATAbox-sub were transformed into *Escherichia coli dut ung* mutant RZ1032, and single-stranded uracil-containing DNA was prepared as previously described (54). Mutagenesis was performed essentially as previously described (35). Briefly, 1 μg of single-stranded DNA was annealed to a 10-fold molar excess of a mutagenic oligonucleotide (see below) by slow cooling, and the primer was extended by incubation with Klenow fragment, T4 DNA ligase, and the four deoxynucleotides at 37°C for 2 h. The DNA was then transformed into *E. coli*, and the resulting clones were sequenced by the dideoxy method by using Sequenase (U.S. Biochemicals). The mutant alleles of *SNR6* were cloned into yeast shuttle vector pSE358 (derivative of pUN10; reference 18) by isolating the *Eco*RI-*Sph*I fragment from each allele and ligating it into *Eco*RI-*Sph*I-cut pSE358.

Oligonucleotides. The following oligonucleotides were used: ΔAB6, 5′-GGAACTGCTGATCACCAAATGTCCACG; Δ138-221, 5′-CGCGAGACAATTTTCGATACTTCACTCG; Δ138-179, 5′-CATACAGGAAGATGGATCCTACTTCACTCG; Δ180-221, 5′-CGCGAGACAATTTTGATACACTGCTG; A-sub, 5′-GTTTCAAATTGAAACAATGTCCACG; A1-sub, 5′-GACCAAATGTAACCGAAGGGTTAC; A2-sub, 5′-CTC TGTATTGTTAACAATTGACCAAATG; AATA-sub, 5′-CG AAAAAAACATTGCGGTATAGTAGCCGAAAATAG; IgBB, 5′-CGAAATAAATCTCGGAGTCGAACGGTTCAT CC; In-sub, 5′-CGCGAACACTATCAAGCGAAAAAAAC; PSE-sub, 5′-CCGAAAATAGTGGAATCCCGCAGGGTAC CACTGTTCATG; TATA-sub, 5′-CGAAAAAAACATTTA TTGCGCGTAGCCGAAAATAG; TATAbox-sub, 5′-CGA AAAAAACATTGCGGGCGCGTAGCCGAAAATAG; 6B, 5′-TCATCCTTATGCAGGG; 6C, 5′-AAAACGAAATAAA TCTC; 14C, 5′-CACAATCTCGGACGAATCCTC.

In vitro transcription. The *S. cerevisiae* subcellular extract was prepared from multiply protease-deficient strain BJ926 (27) by the method of Evans and Engelke (19). Unless otherwise indicated, 22.5-μl transcription reaction mixtures contained 40 mM HEPES (pH 7.9), 65 mM $(NH_4)_2SO_4$, 7 mM $MgCl_2$, 3 mM dithiothreitol, 100 ng of plasmid DNA, and 3 μl of the subcellular extract (36 μg of protein). After incubation for 15 min at 25°C, 2.5 μl of a nucleoside triphosphate mixture (6 mM each ATP, CTP, and UTP and 250 μM [α-^{32}P]GTP [8 μCi] in transcription buffer) was added and incubation was continued at 25°C for another 15 min. Under these conditions, approximately 0.5 transcript per gene was produced. The efficiency of *SNR6* transcription could be increased threefold by increasing the final GTP concentration from 25 to 600 μM. Transcription was terminated, and transcripts were purified as previously described (5) and run on a 6% polyacrylamide–8.3 M urea gel. Gels were exposed to X-Omat AR5 film (Kodak) with or without a Cronex Lightning-Plus intensifying screen (DuPont).

For preparative transcription reactions, nucleoside triphosphates were added with the extract and incubation was for 60 min. Gels were exposed for 1 h with an intensifying screen at 4°C, the bands of interest were excised, and the RNA was eluted by soaking the gel slice in 600 μl of 0.5 M ammonium acetate–0.1% sodium dodecyl sulfate–0.1 mM EDTA–17 μg of *E. coli* tRNA per ml at 37°C for 6 to 24 h. The eluted RNA was then precipitated with 2.5 volumes of ethanol at −20°C. RNase T_1 fingerprinting was done as described by Branch et al. (4).

DNase footprinting. DNA probes for footprinting were made by digesting plasmids pCS6 and pPC1 with *Hin*dIII and *Eco*RI, generating a 432-bp fragment (−120 to +312) containing the U6 gene and a 387-bp fragment (−92 to +285, relative to the mature 5′ end) containing the $tRNA_3^{Leu}$ gene, respectively. The *Hin*dIII sites were filled in with the Klenow fragment of DNA polymerase I, dGTP, dCTP, dATP, and [α-^{32}P]dTTP (3,000 Ci/mmol; Amersham) to make 3′-end-labeled probes, or the *Eco*RI sites were treated with alkaline phosphatase and labeled with T4 polynucleotide

kinase and [γ-^{32}P]ATP (7,000 Ci/mmol; ICN) to make 5'-end-labeled probes. In a footprint assay, 3 μl of yeast subcellular extract was added to an end-labeled DNA probe (15,000 cpm Cerenkov) and 500 ng of pUC118 or specific competitor plasmid DNA in 21 μl of FP buffer [40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) (pH 8.0), 47 mM $(NH_4)_2SO_4$, 7 mM $MgCl_2$, 3 mM dithiothreitol] and incubated at 20°C for 30 min. A 1-μl aliquot of DNase I (BRL), freshly diluted to 8 ng/μl (for the no-extract control) or 165 ng/μl (for reactions with extract) from a 1-mg/ml stock in FP buffer containing 0.5 mM $CaCl_2$ and 100 μg of bovine serum albumin per ml, was added, and digestion was stopped 2 min later by adding 25 μl of 10 mM Tris (pH 8)–20 mM EDTA–0.2% sodium dodecyl sulfate–200 μg of denatured sheared fish sperm DNA per ml. Reaction mixtures were heated at 90°C for 3 min, extracted once with 1:1 phenol-chloroform, and precipitated with ethanol; the DNA fragments were analyzed on a denaturing 6% polyacrylamide gel (20:1 acrylamide-bis, 8.3 M urea, 100 mM TBE [42]). A+G ladders of the footprint probes were generated by the procedure of Maxam and Gilbert (42).

Analysis of in vivo gene function. Mutant alleles of *SNR6* cloned into pSE358 (*CEN4 ARS1 TRP1*) were tested for the ability to function as the sole U6 RNA gene in the cell by transformation (25) into a yeast strain containing an insertion in (DAB017; reference 7), or a replacement of (MWK023), the chromosomal copy of *SNR6*. The latter strain was constructed by insertion of a 2.2-kb *Sal*I-*Xho*I fragment of the yeast *LEU2* gene into *Bsm*I-cut p-539H6, digestion of the resulting plasmid (pΔBsmLEU2) with *Pvu*II, and transformation of the linear fragments into yeast strain PJ43-2b (26), which had previously been transformed with the −39D6 allele of *SNR6* cloned into *Eco*RI-*Hin*dIII-cut YCp50 (*CEN4 ARS1 URA3*). Replacement of positions −316 to +306 of the chromosomal copy of *SNR6* with the *LEU2* gene was verified by Southern blot analysis. The replacement strain was used to determine whether sequences that code for the downstream transcripts are essential for normal cell growth. Trp$^+$ transformants of DAB017 and MWK023 were streaked on plates containing 5-fluoroorotic acid, which selects for loss of the *URA3* plasmid carrying the wild-type allele of *SNR6* (50). Strains that live on 5-fluoroorotic acid plates contain functional *SNR6* alleles on the *TRP1* plasmid; those that die do not. *SNR6* alleles that were found to be lethal and would be expected to give full-length U6 RNA were transformed into a yeast strain containing a pseudo-wild-type allele on YCp50 as its only copy of *SNR6*. Pseudo-wild-type *SNR6* has the first 25 bp of the coding region replaced with the first 12 bp of the RNA coding region of the *Schizosaccharomyces pombe* U6 gene (41) and was a kind gift from Rémy Bordonné and Christine Guthrie. Thus, the pseudo-wild-type U6 RNA gives a shorter cDNA in primer extension reactions, which can be distinguished from the cDNA made from transcripts of mutant *SNR6* genes. Total cellular RNA was prepared from yeast strains by the guanidinium thiocyanate method (59). Primer extension with ^{32}P-labeled oligonucleotides 6C and 14C using 4 μg of RNA was performed as previously described (7), except that dideoxynucleotides were omitted, the annealing incubation was skipped, and extension was carried out at 52°C for 15 min. The products were run on a 6% polyacrylamide–8.3 M urea gel, and the gel was exposed to X-Omat AR5 (Kodak) film for 4 days with an intensifying screen.

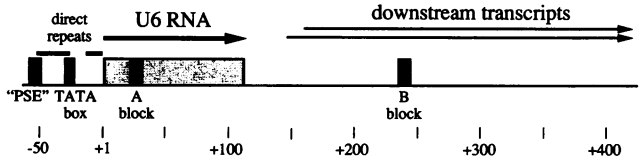

FIG. 1. Positions of potential yeast U6 RNA gene promoter elements. A 500-bp stretch of chromosomal DNA encompassing *SNR6* (7) is shown schematically. The gray rectangle represents the coding region of *SNR6*, and the arrow above it indicates the direction of transcription. The location and orientation of two of the downstream transcripts (see text) are shown by the longer arrows. Potential promoter elements are demarcated by black boxes or bars and are discussed in the text. The scale below the diagram is in base pairs and marks distances from the U6 RNA start site.

RESULTS

Transcription of the wild-type yeast U6 RNA gene in a subcellular extract. For in vitro transcription of *SNR6*, we switched from using a whole-cell extract of *S. cerevisiae* (30) to a subcellular extract (19) that is much more active for both U6 and tRNA gene transcription. The molar yield of transcript per gene in the subcellular extract was approximately 10 times lower for *SNR6* than for a yeast $tRNA_3^{Leu}$ gene (when the concentrations of the initiating nucleotides were equal; see Materials and Methods). The relative activities of previously tested *SNR6* 5' and 3' deletion constructs are the same in the subcellular extract and the whole-cell extract (7; data not shown).

DNA downstream of *SNR6* also gives rise to transcripts in the cell-free system (see, for example, Fig. 5B). These transcripts were found to require the downstream B block for their synthesis and were tentatively mapped on the basis of size and the location of their putative terminator (Fig. 1) (7). The locations of the 5' ends of the downstream transcripts, determined by primer extension of gel-purified transcripts (20b), imply heterogeneous initiation between positions +129 and +177 and termination in an oligo(dT) stretch at position +420. The combined downstream transcripts are approximately equimolar to U6 RNA after 15 min of transcription under standard conditions and most likely arise from the interaction of TFIIIC bound to the B block with DNA sequences that are the optimal distance upstream. Such aberrant initiation has also been observed in an adenovirus VARNA1 gene with artificially increased A-to-B-block spacing (10). The downstream transcripts are not detectable in total cellular RNA, and the DNA that codes for them (excluding the downstream B-block region) is dispensable in vivo (20a).

After 30 min of transcription of *SNR6* in the subcellular extract, an RNA about 90 nucleotides (nt) long began to accumulate in the in vitro reaction, and by 1 h it exceeded U6 RNA in amount (Fig. 2A). The sum of U6 RNA and 90-nt RNA increased linearly during 1 h of incubation, suggesting that both are products of the U6 gene. RNase T_1 fingerprinting analysis showed that the 90-nt transcript is a 3'-truncated form of U6 RNA (data not shown). Incubation of gel-purified full-length U6 RNA in the subcellular extract for various amounts of time revealed that the 90-nt RNA was formed by nucleolytic cleavage of U6 RNA (Fig. 2B). The presence of the intermediate-length products at early time points indicates that the nuclease cleaves U6 RNA progressively in a 3'-to-5' direction. The pause at position +90 may be due to the presence of a stem-loop structure that blocks further

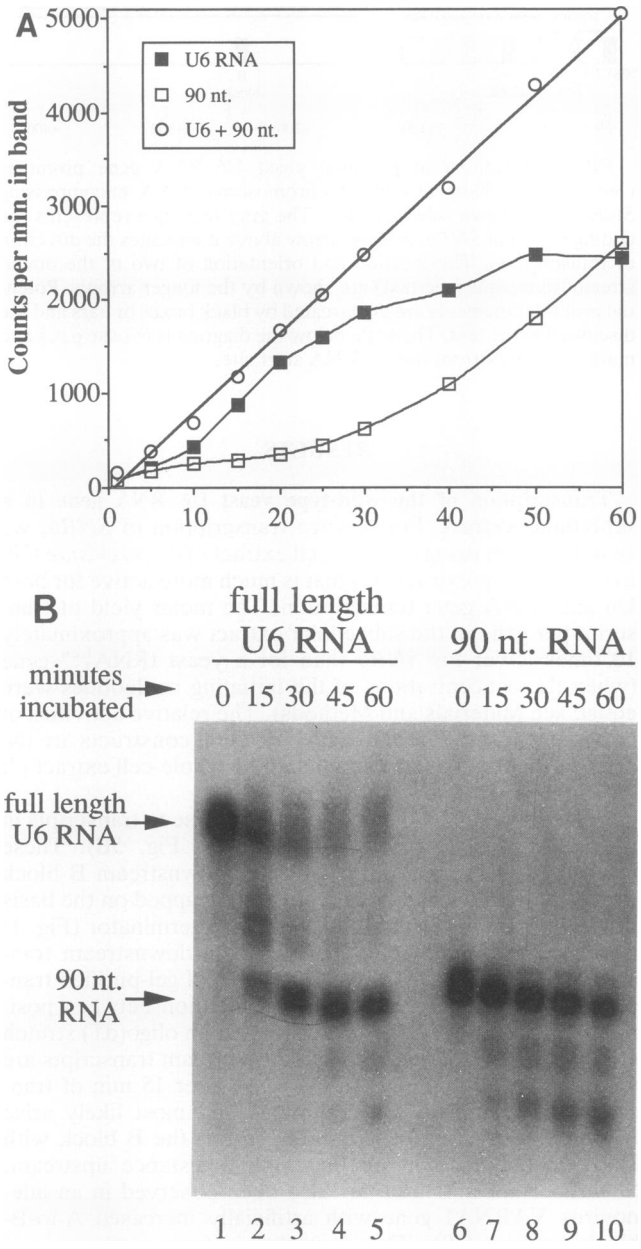

FIG. 2. Identity of the 90-nt RNA. (A) Rate of accumulation of U6 and 90-nt RNAs in the cell-free system. Transcription of *SNR6* (p-539H6) was studied as described in Materials and Methods, except that nucleoside triphosphates were added before the extract, incubation was terminated at the indicated times, and only one-half of each reaction was loaded on the gel. Radioactivity present in the gel at the positions of the U6 (■) and 90-nt (□) RNAs was determined by excision of bands and liquid scintillation, and the value minus the background from the same region of a lane containing pUC118 as a template is graphed. The sum of U6 and 90-nt RNAs at each time (○) is also plotted. The calculated transcript yields from 50 ng (17 fmol) of the plasmid after 60 min were 10 fmol of U6 RNA and 12 fmol of 90-nt RNA. (B) Stability of U6 and 90-nt RNAs in subcellular extract. U6 (lanes 1 to 5) and 90-nt (lanes 6 to 10) RNAs, purified as described in Materials and Methods, from a threefold, 1-h transcription reaction, were each added to mock threefold transcription reactions lacking DNA and [α-^{32}P]GTP. Aliquots (13 μl) were removed at the indicated times, and the RNA was analyzed as for transcription reactions.

digestion (52a). The nuclease activity we observed is likely the same as that seen by Margottin et al. (43), although they did not report which end of U6 RNA is removed or whether the nuclease cuts at a single site or is progressive.

DNase protection of the downstream B block. To test the hypothesis, supported by transcription competition studies (7), that the U6 gene downstream B block binds TFIIIC, *SNR6* was subjected to DNase footprinting analysis. In the presence of a subcellular extract, the protection pattern over the downstream B block was very similar in size to that seen over an intragenic tRNA gene B block, and both displayed specific internal cleavage sites at analogous positions (Fig. 3B; compare lanes 2 and 3 with lanes 11 and 12; summarized in Fig. 3C). Furthermore, *SNR6* constructs competed for the tRNA and U6 RNA gene B block footprints in a similar fashion. An intact U6 gene competed well (lanes 4 and 13), while U6 genes with either a 2-bp deletion or a 7-bp substitution in the B block did not compete (lanes 5, 7, 14, and 16). Likewise, the tRNA gene competed for the tRNA and U6 gene B-block footprints (lanes 6 and 15). Substitution of DNA to within 3 bp of the 3′ border of the *SNR6* B block decreased its ability to compete for the footprinting factor (lanes 8 and 17) and to compete with a yeast 5S rRNA gene for a common transcription factor (7), consistent with the known interaction of TFIIIC with nucleotides outside of the 11-bp core (2, 9; Fig. 3C). (Competitor g is discussed below.) These results strongly support the notion that the U6 gene downstream B block binds TFIIIC.

TFIIIC also binds to the A-block promoter element in tRNA genes, and we saw the expected footprint on the $tRNA_3^{Leu}$ gene in the presence of a subcellular extract (Fig. 3B, lane 12; data not shown). We saw no such footprint over the U6 gene A-block consensus match at positions +21 through +31, suggesting that only a fraction of the TFIIIC molecules bound to the downstream B block also bound to the *SNR6* A block. The remainder presumably bound to the downstream transcription units or solely to the B block. A similar absence of an A-block footprint was observed on a $tRNA_3^{Leu}$ gene with an artificially increased A-to-B block separation (20).

Role of the downstream B block in vivo. We presumed that the lethal phenotype of mutations in the downstream B block (7) is due to decreased synthesis of U6 RNA, as seen in vitro. However, the formal possibility remained that U6 RNA synthesis in vivo is not affected by B-block mutations and the lethality is due to some other function of the sequences that were altered. We therefore measured the level of expression of an allele of *SNR6* that lacks the downstream B block. A strain heterozygous for *SNR6* was constructed, such that one allele had a wild-type coding region but lacked the downstream B block, while the other allele contained a shortened but functional coding region (termed pseudo-wild type; reference 41; see Materials and Methods) and an intact B block. With this strain, one can use primer extension to distinguish transcripts synthesized from the two alleles. Analysis of RNA from the heterozygous strain showed that accumulation of wild-type U6 RNA was virtually abolished when *SNR6* lacked a functional downstream B block (Fig. 4, lane 4). Thus, the B block is important for transcription in vivo, as well as in vitro, and the lethal phenotype of B-block mutants is due to inadequate levels of U6 RNA.

Effect of internal deletions and substitutions on *SNR6* promoter activity. In addition to the B-block promoter element, tRNA genes contain an intragenic A-block element. To determine whether the yeast U6 gene contains any internal

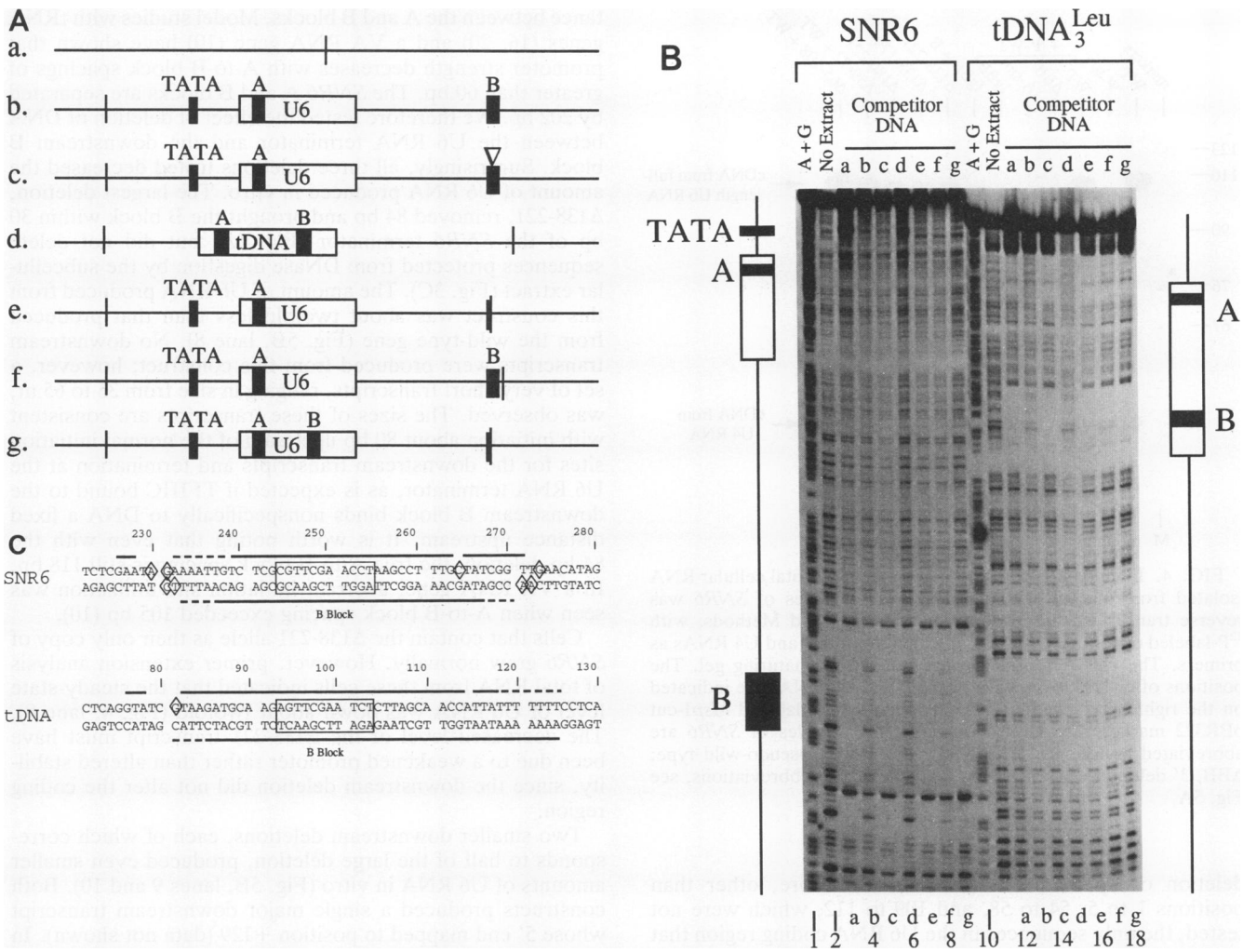

FIG. 3. DNase I footprint of the U6 RNA and tRNA$_3^{Leu}$ genes. (A) Schematic representation of the following plasmid competitor DNAs: a, pUC118; b, pCH6; c, pCH6ΔB; d, tDNA$_3^{Leu}$; e, pCCs6; f, pCD6; g, pIgBB (see Materials and Methods). Gene coding regions are indicated by open rectangles, and the TATA box, A-block, and B-block promoter elements are represented as filled rectangles. Vertical lines represent insert-vector junctions. The triangle represents a 2-bp deletion. (B) DNase footprints and their competition. The 432-bp *SNR6* probe (lanes 1 to 9) and the 387-bp tDNA$_3^{Leu}$ probe (lanes 10 to 18) were 3′ end labeled on the noncoding strand. The A+G ladders (lanes 1 and 10) indicate the positions of purines in the probes. The lanes labeled "No Extract" show the DNA probes treated with DNase I with no extract. The other reactions contained 500 ng of plasmid competitor DNAs a to g and 3 μl of the yeast subcellular extract. The diagrams on either side represent the structures of the footprint probes. (C) Schematic of DNase protection over the B block in both strands of the U6 RNA and tRNA$_3^{Leu}$ genes (compiled from panel B and data not shown). The sequences of the U6 RNA and tRNA genes are shown with the B blocks (rectangles) aligned. The numbering of the tRNA gene is such that +1 corresponds to the 5′ end of the mature tRNA. The solid lines indicate the regions protected from DNase cleavage in the presence of the extract, and the dashed lines indicate regions where protein binding could not be assessed because even naked DNA was refractory to cleavage. The diamonds enclose sites corresponding to enhanced cleavage in the presence of the extract.

promoter elements, such as an A block, we tested the in vitro transcriptional activity of a variety of intragenic deletion constructs, diagrammed in Fig. 5A. A large deletion in the 5′ half of the gene (Δ6-54) resulted in production of only a small amount of transcripts of the expected size (63 to 66 nt; Fig. 5B, lane 3), suggesting that there is an important promoter element in this region. In contrast, a similar-size deletion in the 3′ half of the gene (Δ65-108) resulted in increased transcript production (lane 4). Two sets of Δ65-108 transcripts were seen: the expected 68- to 71-nt products and a smaller set about 60 nt long. Pulse-chase labeling and RNase T_1 fingerprinting analysis showed that the smaller RNAs are processing products of the larger transcripts (data not shown). Presumably, the small RNAs are generated by the same nuclease that converts wild-type U6 RNA into the 90-nt form. The Δ65-108 transcripts accumulate to about four times the level of wild-type U6 RNA, as is true for transcripts of a less extensive 3′ deletion, Δ87-108 (7). It is not clear whether this increased accumulation is due to an increased rate of synthesis, a decreased rate of degradation, or both.

The deleterious effect of the Δ6-54 deletion could be due to removal of the putative A-block element (TGGACATTTGG) at positions +21 through +31. Consistent with this notion, deletion of bp +32 through +53 resulted in increased transcript levels (Fig. 5B, lane 5), as did, to a lesser extent,

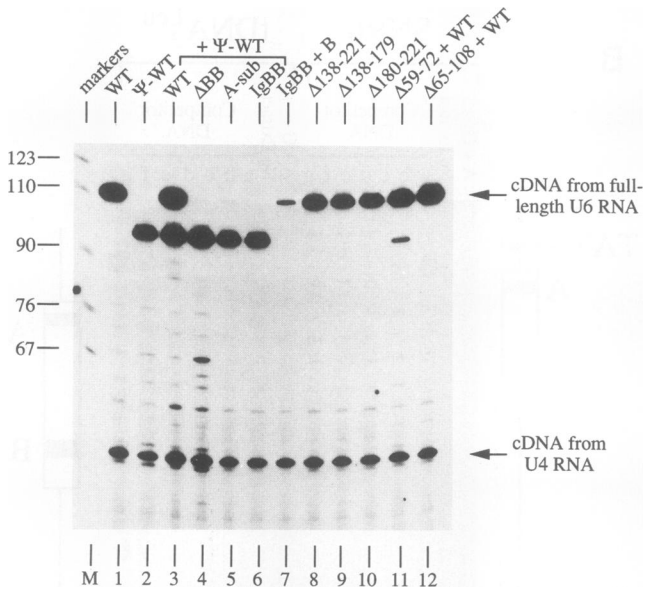

FIG. 4. In vivo expression of *SNR6* mutants. Total cellular RNA isolated from strains bearing the indicated alleles of *SNR6* was reverse transcribed as described in Materials and Methods, with ^{32}P-labeled oligonucleotides complementary to U6 and U4 RNAs as primers. The cDNA products were run on a denaturing gel. The positions of cDNAs from wild-type U4 and U6 RNAs are indicated on the right. The lengths (in nucleotides) of ^{32}P-labeled *Msp*I-cut pBR322 markers are indicated on the left. Alleles of *SNR6* are abbreviated as follows: WT, wild type; ψ-WT, pseudo-wild type; ΔBB, 3′ deletion of B block (pCCs6). For other abbreviations, see Fig. 5A.

deletion of bp 59 to 72 (lane 6). Therefore, other than positions 1 to 5, 54 to 58, and 109 to 112, which were not tested, the only sequences in the U6 RNA coding region that are required for transcript accumulation in vitro lie between positions +6 and +31. As expected from the sizes of the regions deleted and the strong phylogenetic conservation of the U6 RNA structure, none of the transcriptionally active internal deletion constructs can support life in yeast strains lacking wild-type *SNR6* (Fig. 5A). A low level of the Δ59-72 transcript accumulated in vivo, but no Δ65-108 transcript was detected (Fig. 4, lanes 11 and 12). The decreased in vivo level of the internally deleted transcripts relative to wild-type U6 RNA is most likely due to their instability rather than to an in vivo transcription defect.

To test the importance of the A block directly, we substituted its last three base pairs, TGG at positions +29 to +31, with GTT. This three-base substitution (called A-sub) disabled the *SNR6* promoter in vitro (Fig. 5B, lane 7) and inactivated the *SNR6* plasmid in vivo (Fig. 5A). Heterozygotes bearing the A-block substitution allele and a shortened pseudo-wild-type allele accumulated very little of the A-block substitution transcript (Fig. 4, lane 5). The *SNR6* A-block consensus match does, therefore, appear to be an essential promoter element. In agreement with our data, Bordonné and Guthrie found a requirement for an A-block consensus sequence for expression of chimeric human-yeast U6 genes in *S. cerevisiae* (3).

Effect of downstream deletions on *SNR6* promoter activity. The increased accumulation of some of the internally deleted transcripts in vitro may be due, at least in part, to an increase in promoter strength resulting from a decrease in the distance between the A and B blocks. Model studies with tRNA genes (16, 20) and a VA RNA gene (10) have shown that promoter strength decreases with A-to-B block spacings of greater than 60 bp. The *SNR6* A and B blocks are separated by 202 bp. We therefore tested the effect of deletion of DNA between the U6 RNA terminator and the downstream B block. Surprisingly, all three deletions tested decreased the amount of U6 RNA produced in vitro. The largest deletion, Δ138-221, removed 84 bp and brought the B block within 30 bp of the *SNR6* terminator (Fig. 5A) but did not delete sequences protected from DNase digestion by the subcellular extract (Fig. 3C). The amount of U6 RNA produced from this construct was about twofold less than that produced from the wild-type gene (Fig. 5B, lane 8). No downstream transcripts were produced from this construct; however, a set of very short transcripts, ranging in size from 35 to 65 nt, was observed. The sizes of these transcripts are consistent with initiation about 80 bp upstream of the normal initiation sites for the downstream transcripts and termination at the U6 RNA terminator, as is expected if TFIIIC bound to the downstream B block binds nonspecifically to DNA a fixed distance upstream. It is worth noting that even with the 84-bp deletion, the true A-to-B block spacing is still 118 bp. In a VA RNA gene, aberrant initiation site utilization was seen when A-to-B block spacing exceeded 105 bp (10).

Cells that contain the Δ138-221 allele as their only copy of *SNR6* grew normally. However, primer extension analysis of total RNA from these cells indicated that the steady-state level of U6 RNA was down about twofold (Fig. 4, lane 8). The decreased level of the Δ138-221 transcript must have been due to a weakened promoter rather than altered stability, since the downstream deletion did not alter the coding region.

Two smaller downstream deletions, each of which corresponds to half of the large deletion, produced even smaller amounts of U6 RNA in vitro (Fig. 5B, lanes 9 and 10). Both constructs produced a single major downstream transcript whose 5′ end mapped to position +129 (data not shown). In vivo, the 42-bp deletions also resulted in decreased accumulation of U6 RNA, to less than half of the wild-type level (Fig. 4, lanes 9 and 10). Thus, deletion of extragenic DNA between the *SNR6* A block and downstream B block decreased, rather than increased, promoter strength both in vitro and in vivo.

Creation of an intragenic B block. To increase the efficiency of yeast U6 gene transcription and prevent the utilization of alternate (downstream) initiation sites, we sought to introduce a B-block sequence into *SNR6* at a position within the optimal range of spacing from the authentic A block. We noted that a 5-bp substitution could create a perfect match to the downstream B block at positions +85 to +95 of the U6 gene (Fig. 5A, line i) and would give an A-to-B block spacing of 53 bp, within the optimal range for a yeast tRNA gene (1). This mutation (IgBB) was made in an allele of *SNR6* lacking the downstream B block, and its transcriptional activity was tested in vitro (Fig. 5B, lane 11). The intragenic B block did rescue some activity in the absence of the downstream B block, but less than one-third of the wild-type level. As predicted, no downstream transcripts were seen. In combination, the intragenic and downstream B blocks resulted in wild-type levels of U6 RNA and downstream transcripts (Fig. 5B, lane 12). The simplest interpretation of this result is that the downstream B block is used preferentially over the intragenic B block and that the IgBB substitution does not affect transcript stability in vitro.

The construct containing only the intragenic B block is not

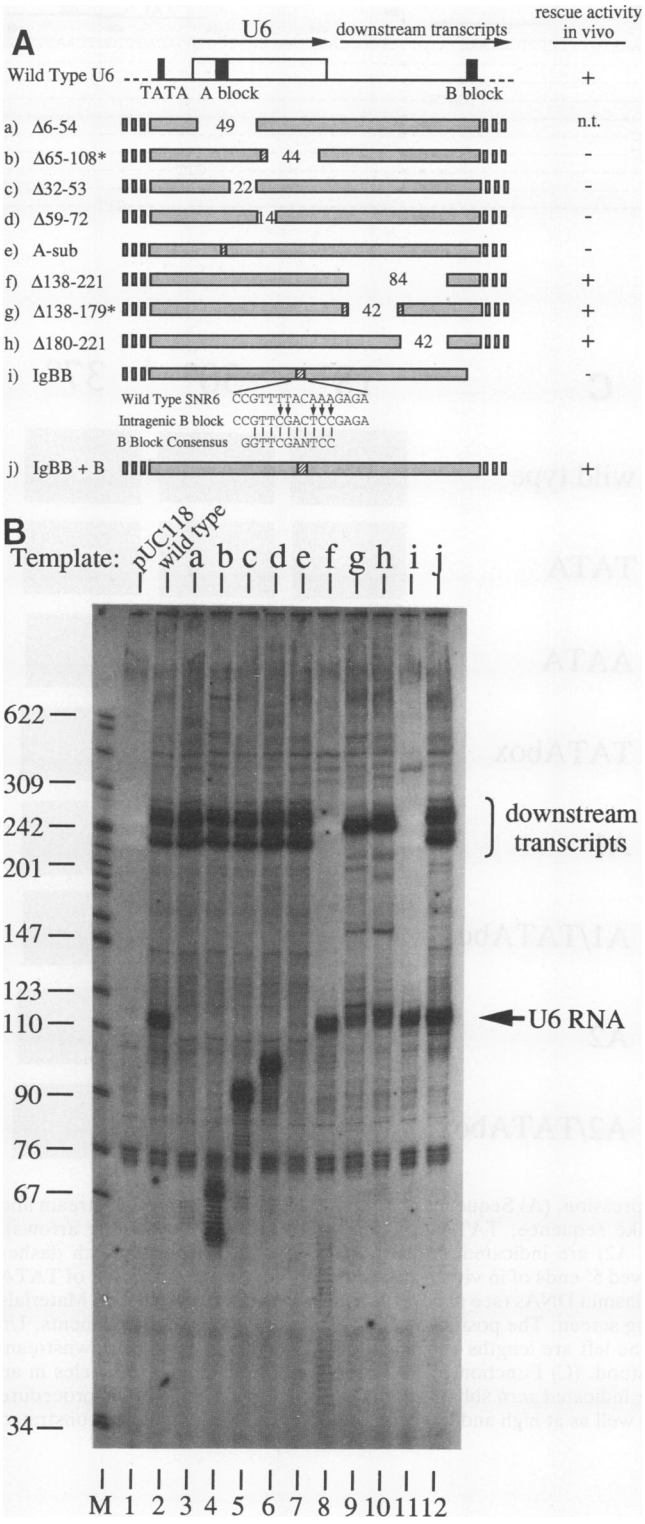

FIG. 5. Effect of internal and downstream deletions and substitutions on expression of *SNR6*. (A) Structures of mutant alleles of *SNR6*. The top diagram depicts wild-type *SNR6*. The RNA coding region is shown as an open box, and black boxes are promoter elements. The gray bars below indicate DNA contained in mutant alleles a to j, with the sizes of the deletions indicated and striped boxes representing substitutions. The ability (+) or inability (−) of each allele to support growth as the sole copy of *SNR6* is indicated at the right (n.t., not tested). An intermediate (i.e., slow-growth) phenotype was not observed for any allele. The two deletion constructs marked with asterisks also contain a small number of base pair substitutions (see Materials and Methods). (B) In vitro transcription of mutant *SNR6* genes. Transcription was done as described in Materials and Methods, with 100 ng of the indicated plasmid DNA (a to j are the mutant alleles depicted in panel A). The positions of downstream transcripts, wild-type U6 RNA, and *Msp*I-cut pBR322 markers (lane M) are indicated. The numbers on the left are lengths in nucleotides.

functional in vivo and produces no stable U6 RNA (Fig. 4, lane 6). Because this mutation required substitution of the coding sequence, the lack of accumulation could be due to instability of the RNA rather than inadequate synthesis resulting from a promoter defect. The intragenic B-block mutation alters three residues that are thought to base pair with U2 small nuclear RNA (24) and one residue important for binding of Prp24, an essential splicing factor involved in assembly of the U4-U6 RNA complex (49). Conceivably, disruption of one or both of these interactions destabilizes U6 RNA. However, the construct with both intragenic and downstream B blocks was functional in vivo (Fig. 5A). Therefore, an RNA defect cannot solely be responsible for the lethality of the intragenic B-block mutation; a transcriptional defect must also contribute. The most likely reason for the poor transcriptional activity of the construct with only the intragenic B block is that sequences outside the 11-bp consensus are nonoptimal for TFIIIC binding. The IgBB allele is unable to compete for B-block footprinting activity (Fig. 3, competitor g), consistent with a defect in binding of TFIIIC. In light of its normal growth rate, it is striking that the strain containing the allele of *SNR6* with both intragenic and downstream B blocks contains less than 10% of the wild-type level of U6 RNA (Fig. 4, lane 7).

Effect of deletion and substitution of upstream sequences. We noted three potential promoter elements upstream of the yeast U6 gene (Fig. 6A). (i) An 11-bp perfect match to positions −42 to −52 of the human U6 gene, which overlap the human PSE (33, 37). (ii) A set of three 13-bp direct repeats with the sequence TTTCG(N)CNACTAT. The first and second repeats are adjacent, while the second and third are separated by 13 bp of an unrelated sequence. (iii) A consensus TATA box conserved in position in all known U6 genes (31). However, an allele of *SNR6* with only 39 bp of yeast upstream DNA (−39H6) and an allele with the PSE-like sequence completely substituted (PSE-sub) are transcribed normally in vitro and in vivo (Fig. 6A; data not shown), indicating the PSE-like sequence and at least one repeat are dispensable. The last 6 bp of the third direct repeat are also found immediately upstream of the initiation site of both the repeated and variant yeast 5S rRNA genes (45, 53), but substitution of this hexamer (In-sub, Fig. 6A) also had no effect on transcription in vitro or in vivo (data not shown). Furthermore, substitution of the first 5 bp of all three repeats had no discernible effect in vitro or in vivo (20c). Thus, we have no evidence for a role of the PSE-like sequence or the direct repeats in transcription of *SNR6*.

To examine the role of the *SNR6* TATA box in promoter function, we generated alleles in which the first four, last four, or all eight positions of the TATA box consensus sequence were substituted. These alleles were named TATA-sub, AATA-sub, and TATAbox-sub, respectively (Fig. 6A). The TATA-sub mutation substantially decreased in vitro transcription, while the AATA-sub mutation had little effect (Fig. 6B, lanes 2 and 3). When the two mutations

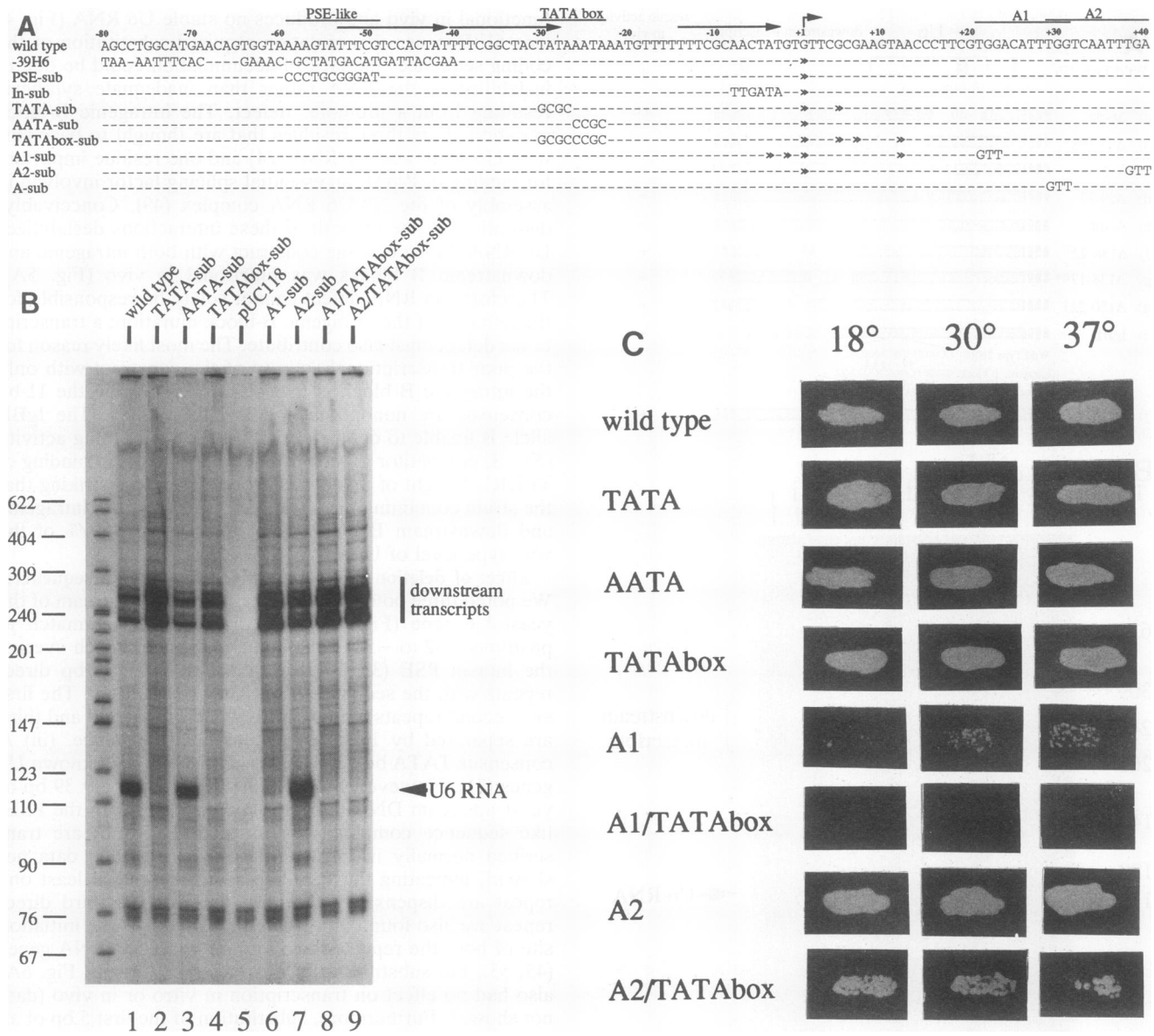

FIG. 6. Involvement of upstream and A-block sequences in *SNR6* expression. (A) Sequences of mutant alleles with altered upstream and A-block sequences. Wild-type *SNR6* is shown at the top. The PSE-like sequence, TATA box sequence, direct repeats (long arrows), transcription start site (short arrow), and A-block sequences (A1 and A2) are indicated. Mutant alleles are shown below, with dashes indicating unaltered positions. The double arrowheads indicate the observed 5′ ends of in vivo transcripts. (B) In vitro transcription of TATA box and A-block mutant alleles of *SNR6*. Transcription of the indicated plasmid DNAs (see panel A) was carried out as described in Materials and Methods. Autoradiography was done for 18 h without an intensifying screen. The positions of ^{32}P-labeled pBR322 *Msp*I fragments, U6 RNA, and the downstream transcripts are indicated. The numbers on the left are lengths in nucleotides. The altered yield of downstream transcripts in the mutant constructs was reproducible and is not understood. (C) Function of TATA box and A-block mutant alleles in an *SNR6* disruption strain. The wild-type *SNR6* gene was replaced with the indicated *snr6* substitution alleles by the plasmid shuffle procedure (see Materials and Methods) at the normal growth temperature (30°C), as well as at high and low temperatures. Growth of cells demonstrated that a given allele can function as the sole copy of the U6 RNA gene.

were combined, no U6 RNA-sized transcript was seen (lane 4). Thus, either the TATA box has an important role in *SNR6* transcription in vitro or the substituted sequence has a repressive effect.

Despite the in vitro results, all three TATA box mutant strains survived with the mutant allele as their only intact copy of *SNR6* and none had an obvious growth defect at 18, 30, or 37°C (Fig. 6C). However, the TATA-sub and TATAbox-sub mutations resulted in the appearance of new 5′ ends in vivo, all of which were downstream of the wild-type start site (Fig. 7a; summarized in Fig. 6A). Approximately 40% of the TATA-sub transcripts started at position +5, with the remainder initiating at the wild-type +1 site (very small amounts of +4 and +3 5′ ends were also seen). The TATAbox-sub allele gave rise to four 5′ ends, in the following proportions: +1, 20%; +5, 35%; +7, 10%; +12, 35%. The normal growth of the TATAbox-sub strain (Fig. 6C) indicates that either the 5′-truncated U6 RNAs

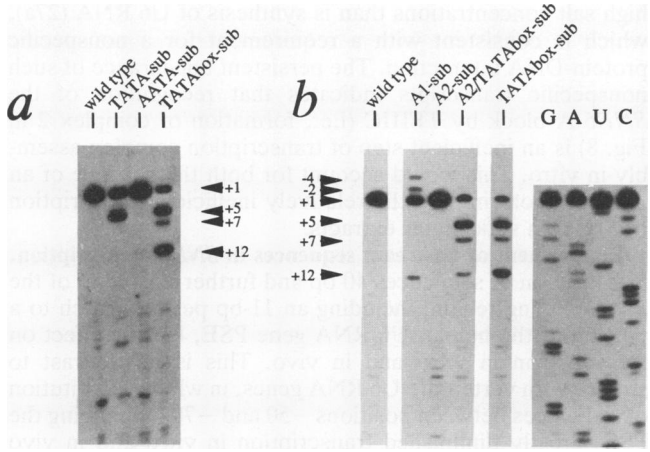

FIG. 7. Primer extension analysis of in vivo transcripts from TATA box and A-block mutant *SNR6* alleles. Total cellular RNAs from the indicated TATA box mutant strains (a) and A-block and double-mutant strains (b) were used as templates for cDNA synthesis from ^{32}P-labeled oligonucleotide 6B (complementary to U6 RNA) as previously described (7), with the following modifications: 2 (a) or 4 (b) μg of RNA was used, and annealing and reverse transcription were done at 52°C. Autoradiography was done for 16 h with an intensifying screen. Lanes G, A, U, and C show a sequencing ladder of wild-type RNA. The positions of primer extension stops relative to the wild-type 5′ end (+1) are indicated with arrowheads.

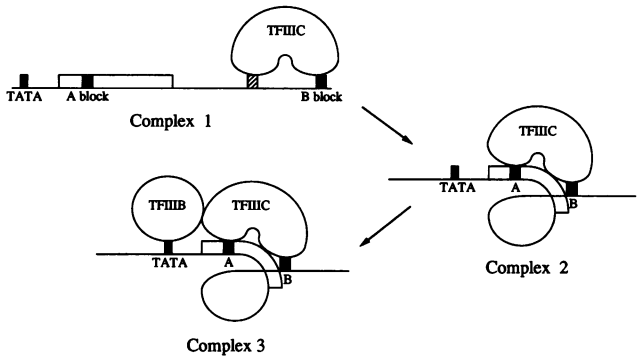

FIG. 8. Model of the assembly pathway of the *SNR6* transcription complex. TFIIIC binds strongly to the downstream B block and weakly (nonspecifically) to a segment of DNA about 45 to 75 bp upstream of the B block (complex 1). Complex 1 isomerizes such that TFIIIC binds to both the A and B blocks (complex 2). TFIIIC then facilitates binding of TFIIIB to the upstream TATA box (complex 3). RNAPIII recognizes complex 3 and initiates transcription of *SNR6*.

retain some function or the reduced amount of the +1 transcript is still in excess of the cell's requirement for U6 RNA.

Cooperation of the TATA box and A block in start site selection. In tRNA genes, the A-block promoter element largely determines the initiation site (reviewed in reference 23). We therefore examined whether, in the absence of the *SNR6* TATA box, the transcription start site is specified by the A block. *SNR6* actually contains two matches to the consensus A-block sequence (TGGCNNAGTGG [22]), starting at positions +21 and +29 (A1 and A2, respectively [Fig. 6A]). On the basis of the structure of tRNA genes, the A1 block is properly located to direct initiation at positions +1 and +5, while A2 has the proper spacing from the +12 site utilized in the TATAbox-sub strain. The lethal A-sub mutation described above (Fig. 4 and 5) alters both the A1 and A2 blocks (Fig. 6A). To test the individual roles of the A1 and A2 blocks in U6 RNA synthesis, the A1-sub and A2-sub mutant alleles were constructed. Consistent with the results obtained with the Δ32-53 deletion (Fig. 5), the A2-sub mutation had no marked effect on U6 RNA synthesis in vitro (Fig. 6B, lane 7), on cell viability when present as the sole copy of *SNR6* (Fig. 6C), or on the U6 RNA level or 5′ end in vivo (Fig. 7b). However, a strain carrying an A2–TATAbox-sub double-mutant allele of *SNR6* made much less of the +12 form of U6 RNA than did the TATAbox-sub strain (Fig. 7b), consistent with a role of the A2 block in initiation at position +12. The A2–TATAbox-sub strain also grew poorly (Fig. 6C), indicating that the combined effect of the A2-sub and TATAbox-sub mutations is sufficient to make U6 RNA rate limiting for growth. Slow growth of the A2–TATAbox-sub mutant strain may be due in part to substitution of two nucleotides (U38 and A40) implicated in the binding of Prp24 (49).

The A1-sub allele was only weakly transcribed in vitro (Fig. 6B, lane 6) and functioned very poorly in vivo (Fig. 6C). Primer extension analysis revealed that A1-sub U6 RNA has heterogeneous 5′ ends (Fig. 7b). A small fraction of the A1-sub U6 RNA had the +12 5′ end, presumably owing to utilization of the A2 block. More interestingly, 5′ ends upstream of +1, at positions −2 and −4, were also observed. These upstream starts may be directed by the TATA box, although we could not test this hypothesis directly because the A1–TATAbox-sub double-mutant strain was not viable (Fig. 6C). Nevertheless, our results indicate that both the A1 block and the TATA box contribute to start site selection in the wild-type yeast U6 RNA gene. In the absence of the TATA box, a cryptic A block (the A2 block) can also direct initiation.

DISCUSSION

Involvement of TFIIIC in *SNR6* transcription. We previously presented a simple model of the structure of the yeast U6 RNA gene transcription complex (7) based on analogy to the well-studied tRNA gene transcription complex. In the U6 gene model, TFIIIC binds to the downstream B block and an intragenic A block and positions TFIIIB over the TATA box upstream of *SNR6* (Fig. 8). Several lines of evidence presented here support the involvement of TFIIIC in yeast U6 RNA gene transcription: (i) DNase protection analysis showed that the *SNR6* downstream B block binds factors present in a yeast cell-free transcription system, and the observed footprint was very similar to that seen over a tRNA gene B block. Furthermore, the *SNR6* B block footprint was competed for by an excess of the tRNA gene, and the tRNA gene B-block footprint was competed for by excess *SNR6*, except when the U6 gene had a mutation in the B-block region.

(ii) Analysis of the in vivo products of a U6 RNA gene bearing a partially substituted downstream B block confirmed that the gene was almost completely inactive transcriptionally; thus, the downstream B block is an essential promoter element in vivo. The same mutant allele was transcriptionally inactive in the yeast subcellular extract (7) and failed to compete for the *SNR6* or $tRNA_3^{Leu}$ gene footprint (Fig. 3).

(iii) Substitutions within the U6 RNA coding region that

create a match to the B-block consensus partially rescue the in vitro transcription of an *SNR6* allele lacking the downstream B block, consistent with the notion that the downstream B block has the same function as the intragenic B block of tRNA genes.

(iv) Deletion analysis of the *SNR6* coding region demonstrates a correlation between the presence of the putative A-block element and the transcript level. Furthermore, a three-base substitution (A-sub) in the A-block consensus abolished *SNR6* promoter activity in vitro (Fig. 5) and greatly reduced U6 RNA accumulation in vivo (Fig. 4). A strain carrying a different three-base substitution (A1-sub) in the presumptive A block was viable but generated U6 RNA with aberrant 5′ ends (Fig. 7). Because the A-block element is intragenic, it is not clear to what extent the growth phenotypes of and U6 RNA levels in the A-sub and A1-sub mutant strains are due to lack of RNA stability or function rather than a transcriptional defect. Nevertheless, the decreased in vitro transcriptional activity of these alleles and the effect of the A1-sub mutation on initiation in vivo indicate that the yeast U6 RNA gene contains an A-block promoter element.

Use of purified TFIIIC to demonstrate a role for this factor in *SNR6* expression is of questionable value, since transcription of *SNR6* in a highly purified system is known to be TFIIIC independent (43, 46). The B-block dependence we found both in the subcellular extract and in vivo presumably reflects either a lower effective concentration of TFIIIB under these conditions (and thus TFIIIC is required to increase the affinity of TFIIIB for the *SNR6* promoter) or the presence of proteins which prevent TFIIIB from binding in the absence of TFIIIC. Definitive proof of the involvement of TFIIIC in *SNR6* expression awaits genetic analysis of the interactions between the B-block-binding subunit (Tfc3, the gene for which has recently been cloned; reference 36) and the downstream B block.

The effect of deleting DNA between the U6 RNA coding region and the downstream B block was unexpected. Studies in which the A-to-B block distance in a VA RNA gene was artificially increased (10) concluded that promoter strength decreases as the A-to-B block distance increases beyond 60 bp. However, we found that reduction of the *SNR6* A-to-B block spacing from 202 to 118 bp decreased promoter strength twofold. Surprisingly, when this 84-bp deletion was split into two 42-bp deletions, each of the smaller deletions resulted in slightly greater inhibition of transcription (Fig. 4 and 5). One possible explanation for this result is that in the wild-type allele, the A and B blocks are brought together by assembly of the intervening DNA into a nucleosome. The 84-bp deletion would represent one full turn around the nucleosome and so may not be as deleterious to TFIIIC binding as the 42-bp deletions, which represent one half turn around the nucleosome. Juxtaposition of promoter elements by binding of a "phased" nucleosome is thought to occur in the *Drosophila hsp26* gene (17).

Another effect of the downstream deletions is to move the initiation site for the downstream transcripts further upstream, with the shift corresponding roughly to the size of the deletion. This suggests that the start sites of the downstream transcripts are specified primarily by distance from the B block rather than by nearby A-block-like sequences. In other words, it appears that TFIIIB is recruited by a TFIIIC molecule whose A-block-binding domain interacts relatively nonspecifically with DNA that is the optimal distance upstream of the B block (Fig. 8, complex 1). We found that synthesis of the downstream transcripts is more sensitive to high salt concentrations than is synthesis of U6 RNA (27a), which is consistent with a requirement for a nonspecific protein-DNA interaction. The persistent appearance of such nonspecific transcripts indicates that recognition of the *SNR6* A block by TFIIIC (i.e., formation of complex 2 in Fig. 8) is an inefficient step of transcription complex assembly in vitro. This would account for both the absence of an A-block footprint and the relatively inefficient transcription of *SNR6* in subcellular extracts.

Involvement of upstream sequences in *SNR6* transcription. Substitution of sequences 40 bp and further upstream of the *SNR6* coding region, including an 11-bp perfect match to a portion of the human U6 RNA gene PSE, had no effect on transcription in vitro and in vivo. This is in contrast to studies with vertebrate U6 RNA genes, in which substitution of sequences between positions −50 and −70 (containing the PSE) greatly diminished transcription in vitro and in vivo (11, 33, 37, 44). TFIIIB is known to bind the region 10 to 40 bp upstream of the tRNA and 5S rRNA genes (28), while a factor distinct from TFIIIB binds to positions −37 to −79 of the mouse U6 gene (56). Therefore, our data are consistent with the hypothesis that TFIIIB alone binds upstream of *SNR6* and TFIIIB and TFIIIC are the only factors required for expression of *SNR6*.

We previously proposed that TFIIIB may directly recognize the TATA box upstream of the yeast U6 RNA gene, as well as interact with TFIIIC bound to the A block (7). If this is true, one would expect that mutations in either the *SNR6* TATA box or A block could affect the fidelity of transcription initiation. We have demonstrated here that this is indeed the case. Substitution of the TATA box caused a downstream shift of the U6 RNA 5′ end to positions appropriately spaced from two A-block consensus sequences (Fig. 6A). Conversely, substitution of the dominant A-block element (A1) resulted in a partial shift of 5′ ends upstream, closer to the TATA box. These data suggest that positioning of TFIIIB on the wild-type yeast U6 RNA gene is directed by balanced interactions (direct or indirect) with the TATA box and the A block. Such a model must be rationalized with the finding of Moenne et al. (46) that the correct 5′ end is obtained when *SNR6* is transcribed in vitro in the absence of TFIIIC and the downstream B block. Positioning of TFIIIB by the A block is expected to be TFIIIC dependent, so our model predicts upstream starts in the absence of TFIIIC binding. A possible explanation for this apparent contradiction is that the 5′ extensions of U6 RNA created by upstream initiation are unstable in vitro and are degraded to the wild-type start site, which corresponds to the first base of an extremely stable stem-loop structure (6). Indeed, the low steady-state level of 5′-extended U6 RNA relative to the +1 transcript in the A1-sub mutant strain may be due to instability of the 5′ extensions rather than predominantly correct (+1) initiation.

It is not clear whether TFIIIB binds the TATA box directly or through an intermediary factor. TFIIIB is thought to be unable to bind DNA specifically (23), and Margottin et al. (43) found that in vitro transcription of *SNR6* with purified factors requires TBP, as well as TFIIIB. However, it has recently been shown that TBP is a subunit of TFIIIB (8, 12, 29, 39, 40, 52, 57). Because TBP is also required for in vitro and in vivo transcription of class III genes that lack TATA boxes (13, 29, 48, 58) and because a mutated TBP unable to bind a TATA box is active in transcription of such genes (48), the predominant role of TBP in TFIIIB is not recognition of the TATA box. Nevertheless, it may perform this function in genes, like *SNR6*, that do contain a TATA

box. Alternatively, as suggested by Kassavetis et al. (29), *SNR6* transcription may require an additional molecule of free TBP for recognition of the TATA box. Our data do not allow us to distinguish between these possibilities or the possibility that one of the other two subunits of TFIIIB recognize the TATA box. Suppression analysis of TATA box mutations is a potential genetic strategy for identification of the factor responsible for *SNR6* TATA box recognition.

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