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TFIIIB Placement on a Yeast U6 RNA Gene In Vivo Is Directed Primarily by TFIIIC rather than by Sequence-Specific DNA Contacts

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The *Saccharomyces cerevisiae* U6 RNA gene (*SNR6*), which is transcribed by RNA polymerase III, has an unusual combination of promoter elements: an upstream TATA box, an intragenic A block, and a downstream B block. In tRNA genes, the A and B blocks are binding sites for the transcription initiation factor TFIIIC, which positions TFIIIB a fixed distance upstream of the A block. However, in vitro transcription of *SNR6* with purified components requires neither TFIIIC nor the A and B blocks, presumably because TFIIIB recognizes the upstream sequences directly. Here we demonstrate that TFIIIB placement on *SNR6* in vivo is directed primarily by the TFIIIC-binding elements rather than by upstream sequences. We show that the A block is a stronger start site determinant than the upstream sequences when the two are uncoupled by an insertion mutation. Furthermore, while TFIIIC-independent in vitro transcription of *SNR6* is highly sensitive to TATA box point mutations, in vivo initiation on *SNR6* is only marginally sensitive to such mutations unless the A block is mutated. Intriguingly, a deletion downstream of the U6 RNA coding region that reduces A-to-B block spacing also increases in vivo dependence on the TATA box. Moreover, this deletion results in the appearance of micrococcal nuclease-hypersensitive sites in the TFIIIB chromatin footprint, indicating that TFIIIB binding is disrupted by a mutation 150 bp distant. This and additional chromatin footprinting data suggest that *SNR6* is assembled into a nucleoprotein complex that facilitates the TFIIIC-dependent binding of TFIIIB.

RNA polymerase III (Pol III) synthesizes a variety of small, stable RNAs including tRNAs, 5S rRNA, 7SK RNA, and U6 RNA. Sequence-specific transcription initiation by Pol III requires auxiliary transcription factors called TFIIIA (a 5S rRNA gene-specific factor), TFIIIB, and TFIIIC (reviewed in references 18 and 60). TFIIIA and TFIIIC are assembly factors for TFIIIB, which serves as the central Pol III transcription initiation factor (33). It has been shown that TATA-binding protein (TBP) is an essential component of TFIIIB (24, 34, 40, 53, 58) and that it plays a general role in Pol III transcription of both TATA box-containing and TATA-less genes (9, 50, 59).

Most genes transcribed by Pol III, including the 5S rRNA and tRNA genes, have intragenic promoters. The promoters of tRNA genes consist of two conserved intragenic elements called the A and B blocks, which are the binding sites of TFIIIC. These promoter elements are separated by 31 to 93 bp in naturally occurring tRNA genes, with the optimum spacing for in vitro transcription being 30 to 60 bp (1, 14). The first nucleotide of the A block of yeast tRNA genes is located 19 ± 1 bp downstream of the transcription start site. TFIIIC bound to the A and B blocks directs binding of TFIIIB a fixed distance upstream of the start site, spanning positions −10 to −40 (35). Although there are no conserved promoter elements upstream of tRNA genes, the frequent occurrence of AT-rich regions may reflect a preference of TFIIIB for such sequences (reviewed in reference 19). In the case of tRNA and 5S rRNA genes, TFIIIB does not bind DNA stably or specifically on its own; rather, it is dependent on TFIIIC for its placement on the DNA (33). However, once TFIIIB is bound, TFIIIC can be removed from the initiation complex by treatment with heparin or high salt concentrations. Pol III recognizes the TFIIIB-DNA complex and initiates transcription. Redirection of TFIIIB binding by a protein with an overlapping binding site results in a commensurate change in the start site, demonstrating that TFIIIB directly positions Pol III (38).

Unlike vertebrate U6 RNA genes, which have exclusively upstream promoters (reviewed in reference 22), the yeast U6 RNA gene (*SNR6*) has a mixed promoter, consisting of an upstream TATA box, an intragenic A block, and a downstream B block (see Fig. 1). Mutations in either the TATA box or the A block alter start site selection in vivo, indicating that both elements cooperate to direct accurate initiation of transcription by Pol III at +1 (13). Chalker and Sandmeyer (7) reached similar conclusions on the basis of an analysis of mutant *SNR6* alleles in which the TATA box was deleted or the distance between the TATA box and the A block was decreased. Mutational studies by Burnol et al. (6) also identified the TATA box and A block of *SNR6* as important promoter elements in vivo, although they did not assess effects on start site selection. In those three studies, the qualitative and quantitative differences between the roles played by the TATA box and A block in *SNR6* start site selection in vivo were not clearly defined.

The *SNR6* B block promoter element, which is located 200 bp downstream of the A block, is essential for transcription in vivo and in crude extracts (3, 6, 13). As is illustrated in Fig. 1, it has been proposed that TFIIIC binds to the *SNR6* A and B blocks, looping out the intervening DNA (6, 13). TFIIIB is then positioned to bind over the TATA box, and this complex is recognized by Pol III. In contrast, in a highly purified system both TFIIIC and the A and B block elements are dispensable for *SNR6* transcription; TFIIIB and Pol III are the only required components (6, 30, 44, 46). Thus, TFIIIB can bind

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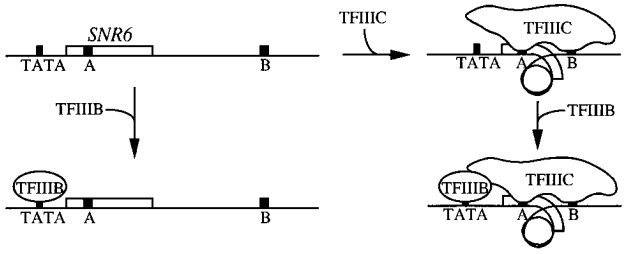

FIG. 1. Model of *SNR6* transcription initiation complex formation. The yeast U6 RNA coding region is indicated by an open box; positions of promoter elements are indicated by black boxes. Two modes of TFIIIB binding are shown: TFIIIC dependent and TFIIIC independent. The former is proposed to also involve DNA folding that is facilitated in chromatin. The latter occurs in vitro, in a highly purified transcription system. See text for details, discussion, and references.

specifically and stably to the *SNR6* promoter in a TFIIIC-independent manner (Fig. 1). However, Sentenac and coworkers have found that after nucleosome reconstitution or chromatin assembly, *SNR6* transcription once again becomes dependent on TFIIIC and the presence of the B block (5). They proposed that binding of TFIIIC to the *SNR6* B block perturbs the local chromatin structure and that this is required for binding of TFIIIB to the upstream sequences.

Here we provide evidence that TFIIIB and TFIIIC function cooperatively via the *SNR6* A block to direct Pol III transcription initiation efficiently and accurately at +1 in vivo. We used a *cis* competition assay, based on an *SNR6* allele with duplicated start sites, to show that the A block plays a stronger role in directing Pol III initiation in vivo than does the TATA box and thus that TFIIIB placement in vivo is primarily directed by protein-protein interactions with TFIIIC. A TATA box mutation that abolishes transcription of *SNR6* in vitro with purified TFIIIB and Pol III affects in vivo U6 RNA gene expression only when the A block is mutated. In vitro transcription with purified components is also sensitive to mutations in a sequence immediately downstream of the TATA box. Chromatin DNase I footprinting established that both TFIIIB and TFIIIC form very similar contacts with *SNR6* in vivo and in vitro. We found that a 42-bp deletion in the U6 RNA gene between the terminator and the downstream B block results in the appearance of micrococcal nuclease (MNase)-hypersensitive sites in and around the TATA box in chromatin and also confers synthetic lethality when combined with certain TATA box mutations. This and the detection of extensive protection from MNase digestion between the *SNR6* A and B blocks in wild-type chromatin suggest that the three-dimensional structure of this extended transcription unit is organized, in chromatin, in such a way as to facilitate the interaction of TFIIIC with its widely separated binding sites.

MATERIALS AND METHODS

Plasmid constructions. The plasmid p-539H6 contains *Saccharomyces cerevisiae* sequence from −539 to +630 relative to the *SNR6* transcription start site and has been described previously (3). Plasmids pΔ138-179 (here called Δ42), pIn-sub, pA1-sub, and pTATAbox-sub were described by Eschenlauer et al. (13). Plasmid p-48/+260 was made by PCR amplification of p-539H6 DNA with oligonucleotides U6-5′Bam and U6-3′Bam. The resulting PCR product was digested with *Bam*HI, gel purified, and ligated into *Bam*HI-cut pUC118 (57), using T4 DNA ligase (U.S. Biochemicals).

Plasmids pstart-dup, pA-29G, pA-29G/T-24C, and pT7-sub were created by oligonucleotide-directed mutagenesis of p-539H6. Plasmids pTATAbox-sub/start-dup and pA1-sub/start-dup were created by oligonucleotide-directed mutagenesis of pstart-dup. Plasmids pTATAbox-sub/Δ42 and pA1-sub/Δ42 were created by oligonucleotide-directed mutagenesis of pΔ138-179. Plasmid pTT TCG-sub was created by oligonucleotide-directed mutagenesis of p-48/+260. Plasmids p-539H6, pstart-dup, pΔ138-179, and p-48/+260 were transformed into *Escherichia coli dut ung* mutant RZ1032, and single-stranded uracil-containing DNA was prepared as previously described (57). Mutagenesis was performed essentially by the method of Kunkel et al. (36). The mutagenized DNA was then transformed into *E. coli*, and the resulting clones were sequenced by the dideoxy method with Sequenase (U.S. Biochemicals).

Plasmid pA1-sub/A-29G/T-24C was generated by digestion of pA1-sub and pA-29G/T-24C with *Nru*I and *Eco*RI, gel purification of the mutation-containing fragments, and ligation with T4 DNA ligase. Plasmid pstart-dup/Δ42 was generated by digestion of pstart-dup and pΔ138-179 with *Eco*NI and *Eco*RI, gel purification of the appropriate fragments, and subsequent ligation.

Each of the *SNR6* mutant alleles was subcloned into the yeast shuttle vector pSE358 (derivative of pUN10 [12]) by isolating the *Eco*RI-*Sph*I fragment from each allele and ligating it into *Eco*RI-*Sph*I-cut pSE358.

Oligonucleotides. Oligonucleotides A1-sub, TATAbox-sub, and 14C have been described previously by Eschenlauer et al. (13). Oligonucleotide U6-D was described by Fortner et al. (16).

The following oligonucleotides were used for site-directed mutagenesis (mutant sequences are underlined): A-29S, 5′-CGAAAAAAACATTTATTTASAG TAGCCGAAAATAG (S = 50% G or C); A-29G/T-24C, 5′-CGAAAAAAA CATTTGTTTACAGTAGCCGAAAATAG; start-dup U6, 5′-GGGTTACTT CGCGAACACATAGTTGCGAACACATAGTTGCGAAAAAAAC; TTTCG -sub, 5′-CACATAGTTGGCTTTAAAACATTTATTTATAGTAGCGCTTTA TAGTGGATCC; and T7-sub, 5′-CATAGTTGCGATCGACCTATTTATTTA TAGTAGCCG.

The following oligonucleotides were used for primer extension or PCR: 6B-iso, 5′-TGCAGGGGAACTGCTGATCATCTCT; 6seq6, 5′-CCAGTAGCAT GAATACTAC; U6-F, 5′-CTAAAATGAATCACGCGG; U6-5′Bam, 5′-CGG GATCCACTATTTTCGGCTAC; and U6-3′Bam, 5′-CGGGATCCGATAGCA AAGGCTTA.

Analysis of in vivo gene function. Mutant alleles of *SNR6* cloned into pSE358 (*CEN4 ARS1 TRP1*) were tested for their abilities to function as the sole copy of the U6 RNA gene by transformation (11) into a yeast strain containing a replacement of the chromosomal copy of *SNR6* (MWK028). Strain MWK028 (*MAT***a** *his3-11,15 leu2-3,112 trp1-1 ura3-52 met2-Δ1 lys2-Δ2 can1-100 ade2-1 Δsnr6*::*LEU2* [YCp50-*SNR6*-ΨWT]) contains a pseudo-wild-type *SNR6* allele (42) on YCp50 (*CEN4 ARS1 URA3* [48]). Leu^+ Trp^+ Ura^+ transformants of MWK028 were tested for growth on 5-fluoroorotic acid (5-FOA), which selects for loss of the *URA3* plasmid (51) that carries the pseudo-wild-type allele of *SNR6*. Strains that survive on 5-FOA contain functional *SNR6* mutant alleles, while those that die do not.

Total cellular RNA was prepared from yeast strains by the guanidinium thiocyanate method (61). Primer extension with ^{32}P-labeled oligonucleotides U6-D and 14C with 2 μg of RNA was performed as previously described (13). The products were resolved in a 6% polyacrylamide–8.3 M urea gel, and the gel was exposed to X-Omat RP film (Kodak) with a Cronex Lightning-Plus screen (DuPont).

Transcription proteins. TFIIIB (purified to the Cibracon blue-Sepharose step), Pol III (Mono Q), and TBP were purified as previously described (30, 33). The amount of TFIIIB is specified in femtomoles of DNA-binding activity assayed on a $tRNA^{Tyr}$ gene, with TFIIIC, as previously described (32); Pol III is specified in terms of femtomoles of active molecules for specific transcription and represents a minimum estimate (35). The concentration of TBP was determined from its extinction coefficient. Analysis of TFIIIB revealed that this material contained trace amounts of TFIIIC.

In vitro multiple-round transcription assay with purified factors. Multiple-round transcription assays were performed essentially as described by Joazeiro et al. (30). Briefly, TFIIIB (0.75 fmol) and TBP (50 fmol) were preincubated with supercoiled DNA template (70 fmol) for 40 min at room temperature (20 to 22°C) in transcription buffer containing 40 mM Tris-Cl (pH 8.0), 7 mM $MgCl_2$, 3 mM dithiothreitol, 25 mM potassium acetate, 35 mM NaCl, 100 μg of bovine serum albumin per ml, and 0.5% (wt/vol) polyvinyl alcohol. The NaCl concentration was raised to 70 mM, and transcription was initiated by adding Pol III (5 fmol) and nucleotides (to a final concentration of 200 μM ATP, 100 μM CTP, 100 μM GTP, and 25 μM [α-^{32}P]UTP at 10^4 cpm/pmol). Transcription was allowed to proceed for 30 min and was stopped by the addition of 6 volumes of stop solution (40 mM Tris-Cl [pH 7.5], 2 mM Na_3EDTA, 0.2% sodium dodecyl sulfate [SDS]) containing a ^{32}P-labeled, 250-bp DNA fragment as a recovery marker. Samples were processed for electrophoresis and analyzed on 8% polyacrylamide denaturing gels (43), and the transcripts were quantified with a Fuji Bio-Imager BAS 1000.

Chromatin footprinting. The yeast strains used for chromatin footprinting contain a single copy of *SNR6* on the chromosome (DAB014 [3] and MWK055 [31]) or on a centromeric plasmid (MWK033 [16] and VG037, VG069, and VG077 [this work]). Yeast strain MWK055 contains *SNR6* with a lethal point mutation in the B block (a T-to-C change at position 237 [T237C]) integrated into the chromosomal locus and a YCp50 plasmid carrying the −39D6 allele (13) of *SNR6*. Only the T237C allele of *SNR6* is detected by multiple rounds of primer extension with an oligonucleotide that binds to sequences that are not present in the −39D6 allele (data not shown). Yeast strains MWK033 and VG077 contain a wild-type copy of *SNR6*, strain VG037 contains *snr6-Δ42*, and strain VG069 contains *snr6-TATAbox-sub*. To obtain VG037, VG069, and VG077, MWK028

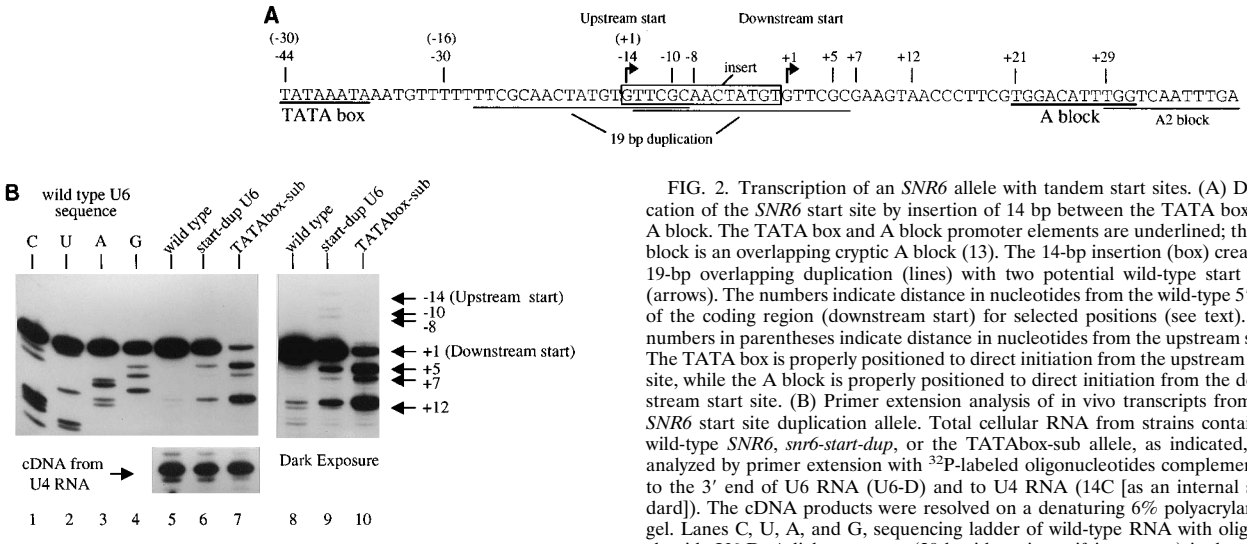

FIG. 2. Transcription of an *SNR6* allele with tandem start sites. (A) Duplication of the *SNR6* start site by insertion of 14 bp between the TATA box and A block. The TATA box and A block promoter elements are underlined; the A2 block is an overlapping cryptic A block (13). The 14-bp insertion (box) creates a 19-bp overlapping duplication (lines) with two potential wild-type start sites (arrows). The numbers indicate distance in nucleotides from the wild-type 5′ end of the coding region (downstream start) for selected positions (see text). The numbers in parentheses indicate distance in nucleotides from the upstream start. The TATA box is properly positioned to direct initiation from the upstream start site, while the A block is properly positioned to direct initiation from the downstream start site. (B) Primer extension analysis of in vivo transcripts from the *SNR6* start site duplication allele. Total cellular RNA from strains containing wild-type *SNR6*, *snr6-start-dup*, or the TATAbox-sub allele, as indicated, was analyzed by primer extension with ^{32}P-labeled oligonucleotides complementary to the 3′ end of U6 RNA (U6-D) and to U4 RNA (14C [as an internal standard]). The cDNA products were resolved on a denaturing 6% polyacrylamide gel. Lanes C, U, A, and G, sequencing ladder of wild-type RNA with oligonucleotide U6-D. A light exposure (20 h with an intensifying screen) is shown on the left side of the panel; a darker exposure of lanes 5 to 7 (4 days with an intensifying screen) is shown on the right. The positions of primer extension stops relative to the wild-type 5′ end (+1) are indicated with arrows on the right.

(see above) was transformed with pSE358-Δ42, pSE358-TATAbox-sub, and pSE358-539H6, respectively, and plated on medium containing 5-FOA to select for loss of the *URA3* plasmid that contains the pseudo-wild-type allele of *SNR6*.

Chromatin DNase I footprinting was performed essentially as described by Hull et al. (27). Briefly, yeast cultures (250 ml) grown in synthetic media were harvested in the exponential growth phase (optical density at 600 nm of 0.5 to 1). Cells were converted to spheroplasts by treatment with Zymolyase 20T (Seikagaku America) for 20 min at 30°C with shaking at 150 rpm. Spheroplasts were lysed in hypotonic buffer and treated for 5 min with 0 to 25 μg of DNase I (Bethesda Research Laboratories) per ml at room temperature. Final concentrations of DNase I are indicated in the figure legends. Yeast genomic DNA was purified by phenol-chloroform extraction, treated with RNase A, and precipitated with isopropanol as previously described (27). We did not digest the purified genomic DNA with a restriction enzyme as recommended because that increased the appearance of artifactual primer extension stops.

DNase I cleavage was detected by multiple rounds of primer extension of purified genomic DNA with ^{32}P-end-labeled oligonucleotides as described by Hull et al. (27). Primer extension reaction mixtures contained 5 μl of DNA (5 to 10 μg), 2 U of *Taq* DNA polymerase (U.S. Biochemicals), and 2 pmol of ^{32}P-labeled oligonucleotide in a 50-μl reaction mixture containing 50 mM KCl, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; adjusted to pH 8.4 with KOH), 2.5 mM $MgCl_2$, and a 100 μM concentration each of dATP, dCTP, dGTP, and dTTP. For oligonucleotides 6B-iso and U6-F, the DNA-primer mix was denatured at 94°C for 30 s, annealed at 45°C for 2 min, and then extended at 72°C for 3 min. Oligonucleotide 6seq6 was annealed at 55 instead of 45°C. Twenty to 30 rounds of primer extension were done. The samples were ethanol precipitated and prepared for electrophoresis as previously described (27). The products were resolved on a denaturing 6% polyacrylamide gel (20:1 acrylamide-bisacrylamide, 8.3 M urea, 100 mM Tris-borate-EDTA [TBE] [43]), and the gel was exposed to X-Omat RP film with an intensifying screen. MNase footprinting was done as described for DNase I footprinting except that the hypotonic buffer used to lyse the cells also contained 1 mM $CaCl_2$. Final concentrations of MNase (Boehringer Mannheim) are indicated in the figure legends.

RESULTS

Transcription of an *SNR6* allele with tandem start sites. To assess the relative contribution of the TATA box and A block to *SNR6* start site selection, we designed a *cis* competition assay based on an *SNR6* allele with tandemly duplicated start sites. Fourteen base pairs of DNA was inserted between positions −1 and +1 of *SNR6*, creating an overlapping duplication of positions −13 to +6 (Fig. 2A). This mutation (called start-dup U6) is expected to uncouple initiation directed by the TATA box and the A block; the TATA box is properly positioned to direct initiation from the upstream start site, while the A block is properly positioned to direct initiation from the downstream start site. The ratio of the steady-state amounts of the transcripts with upstream and downstream 5′ ends should thus reflect the relative influence of the two elements in TFIIIB placement. By leaving intact the sequences immediately flanking the two start sites, any sequence selectivity that might be exhibited by Pol III should not skew start site selection. Furthermore, keeping the tandem start sites close together should prevent TFIIIB from binding upstream of the two sites simultaneously, which presumably would always result in initiation at the downstream site.

When tested for its function in vivo as the sole copy of *SNR6*, the start site duplication allele supported normal cell growth (see below). Total cellular RNA was isolated from this strain, and the *snr6-start-dup* initiation sites were identified by primer extension analysis. The U6 RNA 5′ ends were mapped to positions −14, −10, −8, +1, +5, +7, and +12 relative to the wild-type (downstream) start site, designated as +1 (Fig. 2B, lanes 6 and 9; positions are indicated in Fig. 2A). U6 RNA initiated at the upstream sites was present in a very low abundance relative to U6 RNA with 5′ ends corresponding to downstream start sites. The steady-state level of RNA cannot be taken as a precise measure of the relative utilization of the different start sites, since the relative stability of each RNA species is unknown. However, the fact that U6 RNAs with these same 5′ ends accumulate to much higher levels when synthesized from an *SNR6* allele with a mutant A block (see Fig. 3B, lanes 4) suggests that 5′-extended U6 RNAs are not intrinsically unstable, and thus the relative amounts of *snr6-start-dup* transcripts likely reflect primarily transcriptional effects. Therefore, the A block appears to be a stronger determinant than the TATA box in specifying the Pol III start site on *SNR6* in vivo.

The aberrant downstream initiations at +5, +7, and +12 of the *snr6-start-dup* allele are identical to those seen in the TATAbox-sub mutant, in which the TATAAATA sequence is changed to GCGCCCGC (13) (Fig. 2B, lanes 7 and 10). (We have previously shown that the +12 transcript is directed by a cryptic A block element that overlaps the dominant *SNR6* A block [Fig. 2A, A2 block] [13].) Thus, moving the TATA box and its flanking sequences 14 bp further away from the A block has the same qualitative effect on initiation at the downstream site as does complete substitution of the TATA box. This result is consistent with the notions that the TATA box modifies A

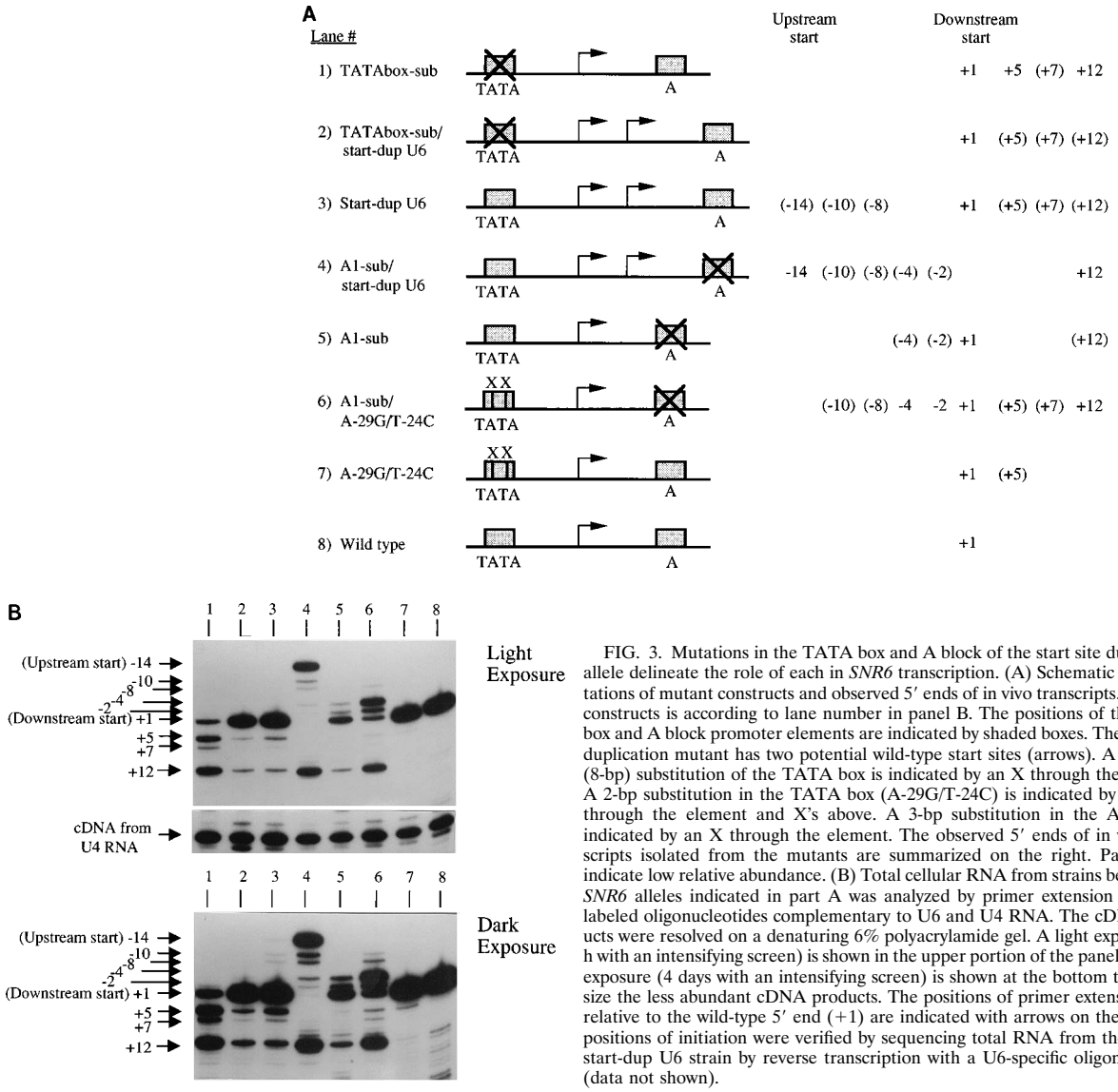

FIG. 3. Mutations in the TATA box and A block of the start site duplication allele delineate the role of each in *SNR6* transcription. (A) Schematic representations of mutant constructs and observed 5′ ends of in vivo transcripts. Order of constructs is according to lane number in panel B. The positions of the TATA box and A block promoter elements are indicated by shaded boxes. The start site duplication mutant has two potential wild-type start sites (arrows). A complete (8-bp) substitution of the TATA box is indicated by an X through the element. A 2-bp substitution in the TATA box (A-29G/T-24C) is indicated by two lines through the element and X's above. A 3-bp substitution in the A block is indicated by an X through the element. The observed 5′ ends of in vivo transcripts isolated from the mutants are summarized on the right. Parentheses indicate low relative abundance. (B) Total cellular RNA from strains bearing the *SNR6* alleles indicated in part A was analyzed by primer extension with ^{32}P-labeled oligonucleotides complementary to U6 and U4 RNA. The cDNA products were resolved on a denaturing 6% polyacrylamide gel. A light exposure (20 h with an intensifying screen) is shown in the upper portion of the panel; a darker exposure (4 days with an intensifying screen) is shown at the bottom to emphasize the less abundant cDNA products. The positions of primer extension stops relative to the wild-type 5′ end (+1) are indicated with arrows on the left. The positions of initiation were verified by sequencing total RNA from the A1-sub/start-dup U6 strain by reverse transcription with a U6-specific oligonucleotide (data not shown).

block-directed initiation and that there are strong positional constraints on TATA box function. Given the large fraction of wild-type +1 RNA present in the start-dup U6 strain, it appears that the T-rich sequence 30 bp upstream of the +1 start (Fig. 2A) is less detrimental to TFIIIB binding than is the GC-rich sequence present at position −30 in the TATAbox-sub allele.

Mutations in the TATA box and A block of *snr6-start-dup* delineate their roles in transcription. In our interpretation of the above results, we assumed that the TATA box directs initiation at the upstream sites and the A block directs initiation at the downstream start sites. To test this assumption, we introduced TATA box and A block substitutions into the *SNR6* start site duplication allele (Fig. 3A). Complete substitution of the TATA box in the start site duplication allele results in the loss of the upstream initiations at positions −14, −10, and −8, while the downstream initiations at positions +1, +5, +7, and +12 are relatively unaffected (Fig. 3B, compare lanes 2 and 3 in the dark exposure). Thus, the TATA box directs only the upstream starts. This result also indicates that upstream initiation does not compete significantly with downstream initiation in the start-dup allele and thus strengthens the notion that the low levels of −14, −10, and −8 RNAs are due to inefficient synthesis rather than rapid degradation. As expected from the amount of wild-type RNA produced, the TATAbox-sub/start-dup strain displayed normal growth.

A 3-bp substitution (called A1-sub) in the A block of *snr6-start-dup* had a dramatic effect: U6 RNA initiated exclusively at upstream (−14, −10, −8, −4, and −2) and aberrant downstream (+12) sites (Fig. 3B, compare lanes 3 and 4). This is a clear manifestation of the intrinsic ability of the upstream sequences to direct TFIIIB binding. Remarkably, the A1-sub/start-dup U6 mutant strain is viable even though it makes no wild-type U6 RNA. Thus, some or all of the aberrantly initiated RNAs are capable of providing the function of U6 RNA. The marked effect of the A1-sub mutation in the start-dup U6 background confirms the dominant role of the A block in start site selection.

Since addition of the 14-bp start-dup insertion to the A1-sub allele results in a commensurate increase in the length of the

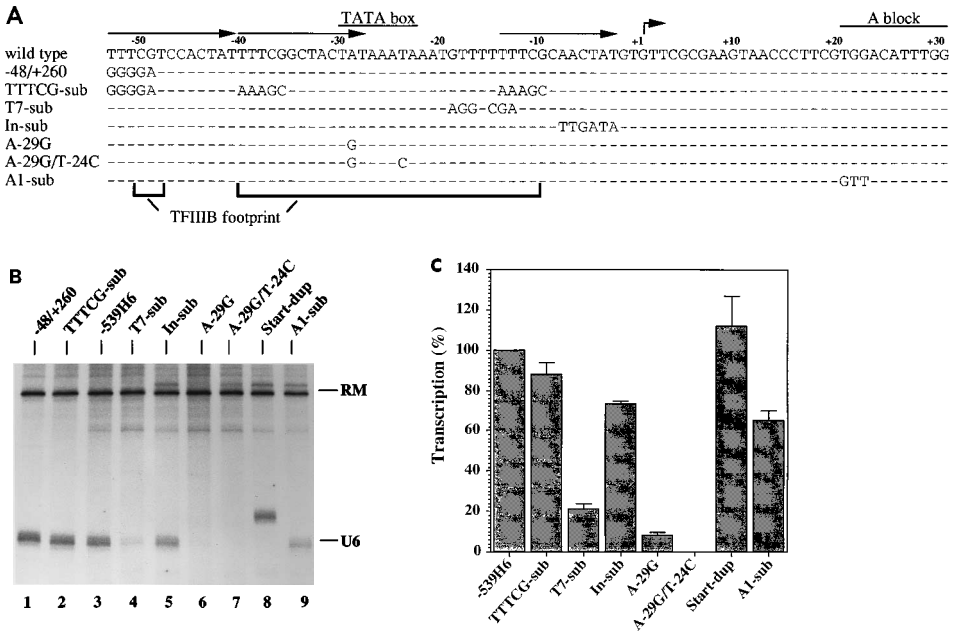

FIG. 4. Transcription of *SNR6* mutant genes with purified components. (A) Substitution mutations tested in vitro. Wild-type *SNR6* is shown at the top. The TATA box sequence, A block, direct repeats (long arrows), and transcription start site (short arrow) are indicated. Mutant alleles are shown below, with dashes indicating unaltered positions. The region of *SNR6* protected from DNase I digestion in vitro with purified TFIIIB is shown at the bottom (30). (B) Multiple-round transcription of *SNR6* mutant alleles with purified TFIIIB and Pol III. The indicated template DNAs (70 fmol) were preincubated with Cibracon blue-purified TFIIIB (0.75 fmol) and recombinant yeast TBP (50 fmol) for 40 min at room temperature. Transcription was initiated by the addition of purified Pol III (5 fmol) and nucleoside triphosphates and proceeded for 30 min. U6 RNA and recovery marker (RM) are identified on the right. Similar results were obtained with TFIIIB reconstituted from recombinant TBP, recombinant Brf1, and a B″ fraction (data not shown). (C) Quantitation of transcription from mutant *SNR6* alleles with purified TFIIIB and Pol III. The U6 transcript was quantified by phosphoimager analysis (Fuji Bio-imager) and was adjusted for sample recovery. The average of three separate experiments is shown. Transcription from pTTTCG-sub is expressed as a percentage of p−48/+260 transcription, while results for the other mutants are expressed as percentages of p-539H6 transcription. The start-dup U6 and A1-sub mutations result in the incorporation of extra labeled nucleotides (five and one, respectively); this was taken into account when quantitating molar yield of transcripts from these templates.

major A1-sub transcript (Fig. 3B, compare lanes 5 and 4), upstream sequences must direct the formation of this transcript. Furthermore, the TATA box is an important part of the upstream element, because a 2-bp substitution that changes the sequence from TATAAATA to TGTAAACA (A−29G/T−24C) redirects A1-sub transcription from +1 to −4, −2, and +12 (Fig. 3B, lanes 6). The same TATA box mutation in the presence of a wild-type A block has virtually no effect (Fig. 3B, lanes 7), again demonstrating the dominant role of the A block in start site selection.

From the above results we can deduce the hierarchical order of the promoter elements that influence *SNR6* start site selection. The A block is the strongest determinant and directs initiation approximately 16 to 20 bp upstream of its 5′ border. In the absence of the A block, upstream sequences assume the role of start site determinant and direct initiation 30 bp downstream of the first T of the TATA box. When both the A block and the TATA box are mutated, a cryptic A block (Fig. 2A, A2 block) efficiently directs initiation at position +12 (13). If the TATA box has a particularly unfavorable sequence (e.g., in the TATAbox-sub allele), the A2 block is highly active even in the presence of the wild-type A block (Fig. 3B, lanes 1). Finally, mutation of the A block and TATA box also results in strong initiation at −2 and −4. The element directing initiation at these sites is not known, but it is interesting to note that the hexamer AACTAT, present at positions −3 to −8 of *SNR6*, is found immediately upstream of both repeated and variant yeast 5S rRNA genes (45, 56). Thus, the *SNR6* promoter may contain a weak initiator element.

Transcription of *SNR6* mutants in a TFIIIC-independent purified system. Clearly, TFIIIB placement upstream of *SNR6* in vivo occurs primarily via a TFIIIC-dependent mechanism (Fig. 1). Nevertheless, the importance of upstream sequences in start site selection when the A block is mutated suggests that sequence-specific TFIIIB-DNA interactions also play a role in vivo. To more directly characterize the upstream elements that TFIIIB recognizes in *SNR6*, we measured the activity of our mutant alleles in a TFIIIC-independent yeast transcription system, utilizing highly purified fractions containing TFIIIB and Pol III (30, 32). In this purified system, TFIIIB placement is expected to be directed solely by DNA binding and should be highly sensitive to mutations in the upstream region. It has previously been shown that 6- to 8-bp substitutions of the TATA box abolish transcription of *SNR6* in a crude yeast subcellular extract (13) or with purified TFIIIB and Pol III (6). To determine the selectivity of TFIIIB binding to the TATA box, we tested more subtle mutations.

To confirm our expectation that TFIIIC-independent initiation is directed solely by upstream sequences, we first analyzed the in vitro transcripts of the start site duplication allele. Only transcripts initiated at the upstream site were observed (Fig. 4B, compare lanes 3 and 8), as is expected if TFIIIB directly recognizes sequences upstream of the start-dup insertion.

As noted in earlier studies (6, 13), sequences more than 48 bp upstream of the start site are not important for *SNR6* transcription, since the −48/+260 construct is transcribed at least as well as the −539/+630 construct (Fig. 4B, compare lanes 1 and 3). A single-base-pair substitution in the TATA box (A-29G), however, nearly abolishes *SNR6* transcription in the

purified system (Fig. 4B, lane 6). Furthermore, the A-29G/T-24C TATA box mutation, shown in Fig. 3 to have almost no effect in vivo (except in combination with the A block mutation), completely abolishes transcription with purified TFIIIB and Pol III (Fig. 4B, lane 7). These results show clearly that TFIIIC-independent transcription of *SNR6* is highly dependent on direct contacts between TFIIIB and specific base pairs in the TATA box. The degree of sequence selectivity is similar to that exhibited by TFIID in binding to a Pol II TATA box (62).

Other data suggest that sequences in addition to the TATA box are important for the TFIIIC-independent function of TFIIIB. When the *SNR6* TATA box was inserted into an analogous position in the *SUP4* $tRNA^{Tyr}$ gene, efficient transcription of the tRNA gene still required the addition of TFIIIC (39). We reasoned that *SNR6* sequences flanking the TATA box might also be important for stable binding of TFIIIB, which is known to protect positions −10 to −40 and −48 to −50 of *SNR6* from cleavage by DNase I in vitro (30). Substitution of the first 5 bp of all three repeats of a 13-bp reiterated sequence spanning the TFIIIB footprint (TTTCG-sub [Fig. 4A]) had little effect on transcript yield (Fig. 4B, lanes 1 and 2), indicating that these sequences are not strong determinants for TFIIIB binding. Substitution of the hexameric sequence conserved in yeast 5S rRNA genes (In-sub) reduced the yield of transcripts only slightly (Fig. 4B, compare lanes 3 and 5). Both the TTTCG-sub and In-sub mutations have been tested previously in vivo and in crude extracts and shown to have no effect on transcript yield (13). Substitution of a region 4 to 10 bp downstream of the TATA box (T7-sub) has almost no effect in vivo (data not shown) but severely reduces transcription with purified components (Fig. 4B, lane 4, and Fig. 4C). This is the first evidence that sequences immediately downstream of the TATA box influence either TFIIIB binding to DNA or subsequent steps of Pol III transcription initiation.

Unexpectedly, transcription of the A1-sub allele is moderately but reproducibly decreased with purified components (Fig. 4B, lane 9). Burnol et al. (6) also observed modest inhibition of *SNR6* transcription in a purified system when the A block is mutated. A possible explanation for these results is that TFIIIB directly contacts the A block. However, because A block mutations are intragenic, the difference in observed transcription level may result from an influence on an early step of RNA chain elongation rather than from an effect on initiation complex formation.

DNase I chromatin footprinting of wild-type *SNR6*. In order to relate the results obtained with the purified transcription system to the expression of *SNR6* in the intact cell, we sought to determine whether TFIIIB contacts the upstream region in a similar fashion in vitro and in vivo. Chromatin footprinting has been used successfully to study transcription complexes assembled in vivo on yeast tRNA genes (25, 27). We adapted this technique to examine *SNR6* transcription complexes. Exponentially growing cells containing a wild-type copy of the U6 RNA gene on the chromosome or on a centromeric plasmid were digested with Zymolyase to create spheroplasts. After the spheroplasts were disrupted and lightly treated with DNase I, the DNA was extracted and precipitated. DNase cleavage sites in the vicinity of *SNR6* were mapped by multiple rounds of primer extension with *Taq* DNA polymerase and an end-labeled DNA primer complementary to sequences in or near the U6 gene. The resulting DNA was resolved on a 6% denaturing polyacrylamide gel and examined by autoradiography.

The DNase I protection pattern over the region encompassing *SNR6* was first analyzed with a primer complementary to the nontranscribed strand downstream of the B block. The results presented in Fig. 5A show that the region encompassing the B block is protected from DNase I digestion in chromatin (compare lanes 3 and 5). Fairly complete protection is seen over bp +228 to +253, and partial protection occurs from bp +221 to +228 and +255 to +262. Unhindered or enhanced DNase I cleavage is seen at bp +218 to 219 and bp +254. The pattern of protection from DNase I cleavage over the B block is similar to that seen in vitro in crude extract (13) and with purified TFIIIC (6). However, the protection is less complete, especially over the sequences flanking the B block. This may indicate that TFIIIC does not remain bound to the B block at all times in vivo or that it undergoes a conformational change. There is also a region of protection from DNase I digestion upstream of the U6 RNA coding region, over the TATA box (Fig. 5A, compare lanes 3 and 5, and see below). Furthermore, there appears to be continuous partial protection of the region upstream of the B block and downstream of the terminator, but not of most of the coding region (Fig. 5A, compare lanes 3 and 5).

To better resolve the protein-DNA interactions upstream of the U6 RNA coding region, the DNase cleavage products were extended with a primer that binds to the nontranscribed strand within the coding sequence (Fig. 5B). Protection from DNase I cleavage over sequences just upstream of the *SNR6* coding region is clearly seen (Fig. 5B, compare lanes 4 and 5). The protection extends continuously from bp −9 to −35, with additional protection from bp −48 to −50. There is enhanced cleavage by DNase I at positions −4 to −8 and −46 to −47. The concordance of the chromatin DNase I footprint over the upstream region and the DNase I footprint obtained on *SNR6* in vitro with highly purified TFIIIB (30) is remarkable and clearly indicates that TFIIIB binds to the upstream region of *SNR6* in chromatin, as it does to DNA in vitro. In chromatin, there also appears to be partial protection of sequences upstream of position −60; substitution of these sequences has previously been shown to be without effect on gene activity in vivo (6, 13).

A primer that binds upstream of the TATA box was used to study protein-DNA interactions on the transcribed strand of *SNR6* (Fig. 5C). Comparison of DNase I digestion of deproteinized DNA (Fig. 5C, lanes 4 and 5) and the cell lysate (Fig. 5C, lane 6) again shows strong protection from cleavage over the *SNR6* TATA box and neighboring sequences. The protection extends from bp −44 to −8 and is flanked downstream by a region of enhanced DNase I cleavage. Purified TFIIIB has not been footprinted on this strand of *SNR6*, but these results are clearly consistent with results from the analysis of the nontranscribed strand. The footprint on the transcribed strand of *SNR6* is also similar to that seen on the transcribed strand of tRNA genes with purified TFIIIB and TFIIIC (35). As noted above, a region of partial protection from DNase I cleavage is also apparent between the end of the coding region and the B block on the transcribed strand of *SNR6* (Fig. 5C, compare lanes 5 and 6).

Decreasing the *SNR6* A-to-B block distance results in synthetic lethality when combined with the TATAbox-sub mutation. We previously demonstrated that a mutation that deletes 42 bp between the U6 coding region and the downstream B block, here called Δ42 (Fig. 6A), results in an approximately twofold decrease in U6 RNA accumulation in vivo, although no growth phenotype was observed (13). Unexpectedly, the Δ42 mutation is lethal in combination with the 8-bp TATAbox-sub mutation, which also does not produce any growth phenotype on its own (Fig. 6B). The effect of the Δ42 mutation is unlikely to be due to loss of a previously unidentified promoter element, since complete substitution of 83 bp of DNA down-

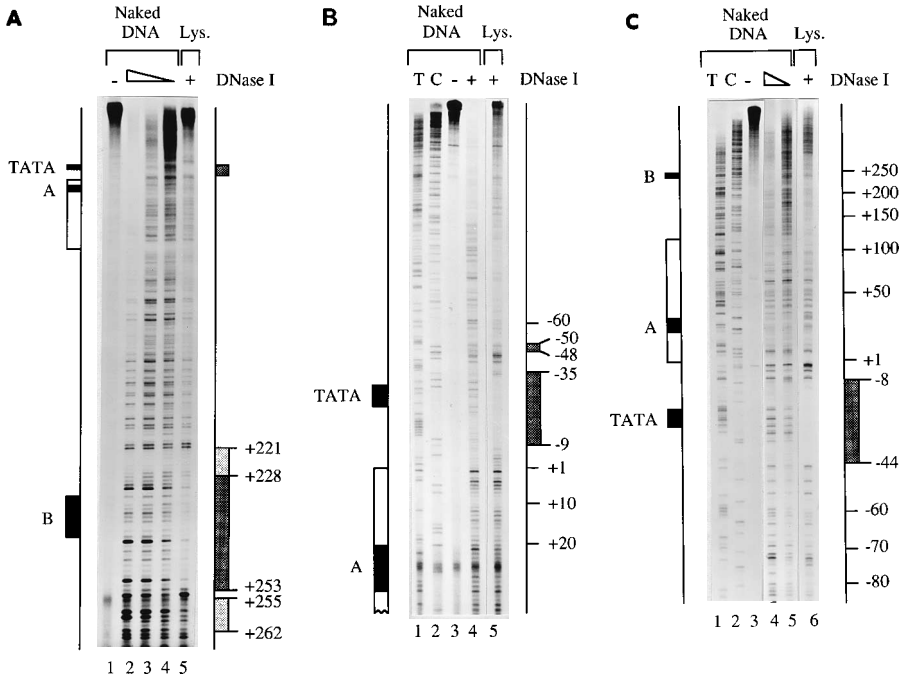

FIG. 5. Chromatin DNase I footprint on wild-type *SNR6*. (A) DNase I protection pattern over the region encompassing *SNR6*. Cell lysate from a yeast strain containing a single copy of *SNR6* on a centromeric plasmid (MWK033) was treated with 25 μg of DNase I (lane 5) per ml. The DNase I digestion pattern was analyzed by primer extension (30 rounds) of purified DNA with ^{32}P-labeled oligonucleotide U6-F, which is complementary to bp +275 to +291 of the *SNR6* nontranscribed strand. Primer extension on undigested genomic DNA (lane 1) was done as a control for pausing by *Taq* DNA polymerase. Genomic DNA was digested with DNase I after purification (with the following amounts of DNase I: lane 2, 12 μg/ml; lane 3, 6 μg/ml; and lane 4, 3 μg/ml) to obtain the pattern of digestion of naked DNA by DNase I. The positions of the U6 coding region and promoter elements are shown on the left and were determined by dideoxy sequencing reactions performed on purified genomic DNA (not shown). The extent of protection from DNase I cleavage in the cell lysate is indicated on the right; darkly shaded boxes indicate regions of strong protection from DNase I cleavage, while lightly shaded boxes indicate regions of partial protection in the vicinity of the B block. (B) DNase I protection pattern over the upstream region of the *SNR6* nontranscribed strand in chromatin. Cell lysate from a yeast strain containing a wild-type chromosomal copy of *SNR6* (DAB014) was treated with 25 μg of DNase I (lane 5) per ml. The digestion pattern was analyzed by primer extension (20 rounds) of purified DNA with ^{32}P-labeled oligonucleotide 6B-iso, which is complementary to bp +49 to +73 of the *SNR6* nontranscribed strand. Lanes 1 and 2, dideoxy sequencing reactions performed on purified genomic DNA; lane 3, primer extension control on undigested genomic DNA; lane 4, purified genomic DNA digested with 3 μg of DNase I per ml. The positions of the U6 coding region and promoter elements are shown on the left. The extent of complete protection from DNase I cleavage in the cell lysate is indicated by shaded boxes on the right. (C) DNase I protection pattern over the upstream region of the transcribed strand of *SNR6* in chromatin. Cell lysate and controls are as described for panel B, except that an additional lane of purified genomic DNA digested with 1.5 μg of DNase I per ml is shown (lane 5). Lane 3 shows that there is a primer extension artifact at position −1, perhaps because of the presence of U6 RNA that escaped digestion by RNase A. The digestion pattern was analyzed by primer extension (30 rounds) of purified DNA with ^{32}P-labeled oligonucleotide 6seq6, which is complementary to bp −143 to −126 of the *SNR6* transcribed strand. Lys., lysate.

stream of the U6 RNA coding region, including the sequences removed by the Δ42 deletion, does not reduce transcript yield in vivo or in a subcellular extract (20). We presume that synthetic lethality occurs because moving the B block prevents efficient binding of TFIIIC to the A block, increasing the reliance of TFIIIB on the TATA box for its proper positioning. However, this increased reliance does not approach that observed with TFIIIC-independent transcription with purified components, since less severe TATA box mutations are not lethal in combination with Δ42 (data not shown). The Δ42 mutation also greatly reduced the viability of the start-dup strain (Fig. 6B). However, some colonies grew, perhaps by amplifying the *snr6* plasmid to a higher copy number; we did not investigate this possibility further. The A1-sub strain, which already grows very poorly (13), was inviable when it also contained the Δ42 mutation (data not shown).

To directly measure the decrease in *SNR6* transcription resulting from the TATAbox-sub/Δ42 double mutation, we analyzed RNA from a heterozygous strain. In this heterozygote, the plasmid carrying the TATAbox-sub/Δ42 allele contains an essential auxotrophic marker as well as a centromere to ensure its stable maintenance. Wild-type *SNR6* function is provided by a shortened but fully active pseudo-wild-type allele (42). In total cellular RNA prepared from a control heterozygous strain that contains the pseudo-wild-type allele and the TATAbox-sub allele without the downstream deletion, the +1, +5, and +7 transcripts from TATAbox-sub are clearly seen (Fig. 6C, lane 5). Likewise, the pseudo-wild-type+Δ42 strain makes large amounts of +1 transcript (Fig. 6C, lane 3). In contrast, in a pseudo-wild-type+TATAbox-sub/Δ42 heterozygote, the +1, +5, and +7 transcripts are barely detectable (Fig. 6C, lane 4), confirming that deletion of downstream sequences confers a transcriptional defect. The pseudo-wild-type+start-dup/Δ42 heterozygote produces a small amount of +1 transcript (Fig. 6C, lane 7), while the pseudo-wild-type+A1-sub/Δ42 heterozygote produces no detectable U6 transcript (Fig. 6C, lane 8). Thus, the growth phenotypes of the double mutant strains correlate well with the amount of U6 RNA transcript produced. Notably, the Δ42 mutation, when combined with other promoter mutations tested, exhibits a synergistic rather than additive effect on the inhibition of *SNR6* transcription, suggesting that the Δ42 mutation directly interferes with promoter element function. Furthermore, the Δ42 mutation decreases the overall level of U6 RNA without affecting the sites of initiation (Fig. 6C and data not shown), indicating that it affects the efficiency but not the position of TFIIIB binding.

MNase chromatin footprinting of wild-type and mutant *SNR6* alleles. The deleterious effects of the Δ42 mutation are

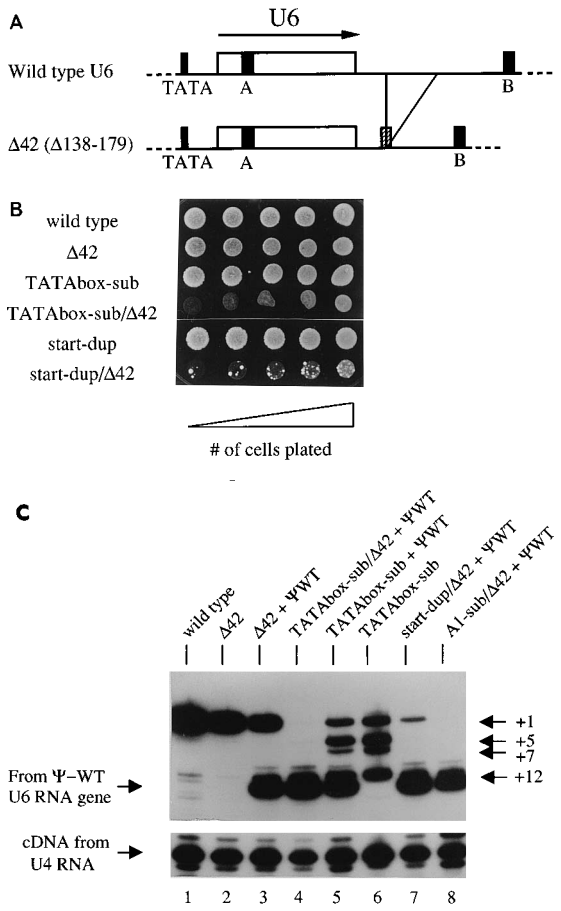

FIG. 6. An *SNR6* mutation that deletes 42 bp of noncoding sequence between the A and B blocks results in synthetic lethality when combined with other promoter element mutations. (A) Structure of the Δ42 mutant allele of *SNR6*. The top diagram depicts wild-type *SNR6*. The RNA coding region is shown as an open box, and black boxes are promoter elements. The bottom diagram shows the position of the Δ42 mutation; this mutation deletes bp 138 to 179 and also contains a small number of base pair substitutions that introduce a *Bam*HI restriction site (indicated by cross hatches). (B) Function of the TATAbox-sub/Δ42 and start-dup/Δ42 mutant alleles in an *SNR6* disruption strain. The wild-type *SNR6* gene was replaced with the indicated *snr6* mutant alleles by the plasmid shuffle procedure. Liquid cultures of the indicated strains were diluted to OD_{600} = 4.5, and successive twofold serial dilutions of each were plated on 5-FOA. Growth of cells demonstrates that a given allele can function as the sole copy of the U6 RNA gene. (C) In vivo expression of *SNR6* Δ42 synthetic lethal mutant alleles. Mutant *SNR6* alleles were introduced into a strain containing a functional pseudo-wild-type *SNR6* allele that makes a 5′-shortened U6 RNA (ΨWT). Total cellular RNA was analyzed by primer extension with ^{32}P-labeled oligonucleotides complementary to U6 and U4 RNA. The cDNA products were resolved on a denaturing 6% polyacrylamide gel. The positions of primer extension stops relative to the wild-type 5′ end (+1) are indicated by the arrows on the right.

surprising, since a decrease in the distance between the *SNR6* A and B blocks (200 bp in the wild-type gene) would be expected to improve TFIIIC binding (the optimal A-to-B block spacing is 30 to 60 bp [1, 14]). One possible explanation is that a nucleosome assembles between the A and B blocks and brings them to within their optimal spacing by spooling in 160 bp of intervening DNA (13) (Fig. 1). In this case, a 42-bp deletion, which corresponds to one half-turn around a nucleosome, might disrupt the alignment of the A and B blocks. Alternatively, nonhistone chromatin proteins or auxiliary transcription factors may bind to this region. To further examine the chromatin structure of *SNR6*, we utilized high-resolution MNase chromatin footprinting. We expected that MNase would be a more sensitive probe of chromatin structure than DNase I, which cleaves more readily within nucleosomes than does MNase. In some of the few published examples of high-resolution MNase chromatin footprinting of suspected nucleosomal regions, the extent of protection is somewhat less than the 146 bp observed in low-resolution in vivo studies (17, 29, 47). In one report, an ≈100-bp stretch of protected DNA is followed by a region of mixed protection and enhancements (47).

We first looked at the chromatin structure of wild-type *SNR6*. Figure 7A shows the results of a primer extension analysis of genomic DNA following MNase digestion of purified DNA (lanes 3 and 4) or of cell lysate containing a wild-type chromosomal copy of the U6 gene (lanes 5 and 6). The oligonucleotide used in the primer extension reaction mixture binds to the nontranscribed strand of *SNR6* downstream of the B block. MNase digestion of purified DNA generates a nonuniform cleavage pattern due to the sequence specificity of the enzyme, which preferentially cleaves AT-rich sequences (15). The MNase digestion patterns of cell lysate and deproteinized DNA are markedly different (Fig. 7A, compare lanes 3 and 5). An interesting pattern of protections and enhancements of MNase cleavage is seen over the entire region encompassing *SNR6*. The B block is protected from MNase digestion to position +218; approximately the same region is protected from DNase I cleavage (Fig. 5A). Most notably, there is nearly complete protection from MNase digestion between the A and B blocks from bp +94 to +198, flanked on either side by enhanced segments of MNase cleavage; as described above, this region is partially protected from DNase I cleavage (Fig. 5A and C). The length of the protected region is similar to what Patterton and Simpson (47) reported for the *S. cerevisiae STE6* promoter. Those authors proposed that weak association of the nucleosomal DNA at the ends of the histone octamer might result in partial accessibility to MNase, producing a footprint of less than the expected 146 bp. The region of *SNR6* protected from MNase does not give well-defined cleavage maxima at 10-bp intervals following DNase I digestion (Fig. 5A, lanes 4 and 5); this suggests that, if a nucleosome is present, it does not have a unique rotational phase (52). Alternatively, the footprint may be due to the binding of a nonnucleosomal protein complex.

Figure 7B shows a comparison of the MNase digestion patterns of wild-type *SNR6* and *snr6-Δ42*, using a primer that binds to the transcribed strand upstream of the TATA box. On the wild-type U6 gene, protection from MNase digestion is seen from bp −43 to −10, with strong enhancement at bp −4 (compare lanes 4 and 5). Given the striking similarity of chromatin DNase I footprints (Fig. 5B and C) with footprints of purified TFIIIB, this protection is almost certainly due to TFIIIB binding. There is also an alternating pattern of MNase enhancement and protection throughout the coding region. As already described for the nontranscribed strand of *SNR6*, there is nearly complete protection from MNase digestion between the A and B blocks from bp +94 to +185 on the transcribed strand. The 42-bp deletion did not reposition the 100 bp of protection between the A and B blocks as might be predicted if a nucleosome were bound between these elements; rather, the size of the MNase-protected segment between the A and B blocks decreased.

Unexpectedly, the MNase digestion pattern of the *snr6-Δ42* upstream region is strikingly different from that of the wild-type gene (Fig. 7B, compare lanes 5 and 6). The Δ42 mutant allele has strong MNase enhancements at bp −43 and −29, minor enhancements at bp −32, −27, and −26, and loss of protection at other positions in the TATA box (Fig. 7B, lanes

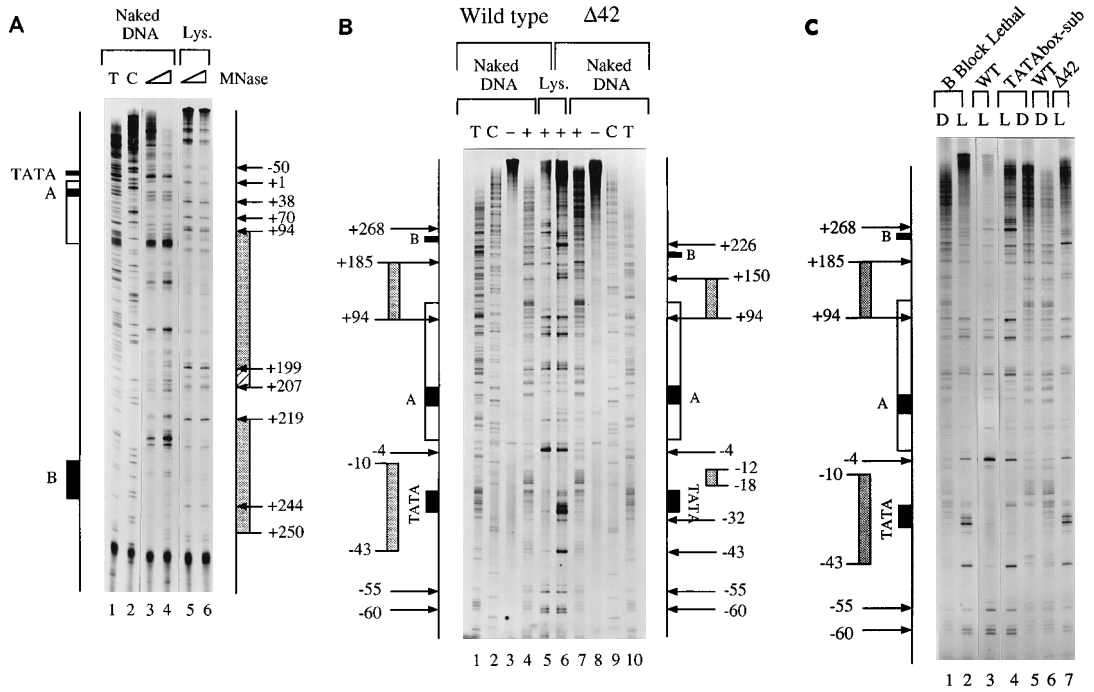

FIG. 7. MNase chromatin footprint of wild-type and mutant *SNR6* alleles. (A) MNase protection pattern over the region encompassing *SNR6*. Cell lysate from DAB014 was treated with MNase at a final concentration of 3,200 U/ml (lane 5) or 6,400 U/ml (lane 6). The MNase digestion pattern of the nontranscribed strand was analyzed by primer extension (25 rounds) of purified DNA with ^{32}P-labeled oligonucleotide U6-F. As a control, purified genomic DNA was digested with MNase at 1.25 U/ml (lane 3) and 2.5 U/ml (lane 4). The positions of the U6 coding region and promoter elements are shown on the left and were determined by dideoxy sequencing of purified genomic DNA (lanes 1 and 2). Shaded boxes show selected regions of protection from MNase cleavage; the hatched box and arrows represent sites of enhanced MNase cleavage. (B) Comparison of MNase protection patterns over the transcribed strand of *SNR6* and *snr6-Δ42*. The MNase digestion pattern of chromatin and naked DNA from strains bearing wild-type *SNR6* (DAB014; lanes 1 to 5) or *snr6-Δ42* (VG037; lanes 6 to 10) was analyzed by 30 rounds of primer extension with ^{32}P-labeled oligonucleotide 6seq6. + and −, the presence and absence, respectively, of MNase. Dideoxy sequencing ladders are shown in lanes 1, 2, 9, and 10; lanes 3 and 8 are controls for primer extension stops on undigested genomic DNA. Purified genomic DNA was digested with 1.25 U of MNase per ml as a control for sequence-specific cleavage by MNase (lanes 4 and 7). DAB014 cell lysate was digested with 3,200 U of MNase per ml (lane 5); VG037 cell lysate was digested with 6,400 U of MNase per ml (lane 6). The positions of the U6 coding region and promoter elements are shown on the left for *SNR6* and on the right for *snr6-Δ42*. Selected MNase protections and enhancements are indicated as described for panel A. (C) Comparison of MNase protection patterns over the transcribed strand of *snr6-T237C*, *snr6-TATAbox-sub*, and *snr6-Δ42*. The MNase digestion pattern of chromatin and naked DNA from strains bearing a lethal B block mutant allele of *SNR6* (MWK055; lanes 1 and 2), wild-type *SNR6* (DAB014, lane 3; VG077, lane 6), *snr6-TATAbox-sub* (VG069; lanes 4 and 5), or *snr6-Δ42* (VG037; lane 7) was analyzed by 30 rounds of primer extension with ^{32}P-labeled oligonucleotide 6seq6. The MNase footprint of the wild-type U6 RNA gene was identical when the gene was on the chromosome or a yeast centromeric vector (data not shown). L, lysate samples (T237C, 4,000 U of MNase per ml; TATAbox-sub, 8,000 U/mL; the wild type and Δ42 were digested as for panel B); D, naked DNA controls (digested with 2.5 U of MNase per ml). The positions of the wild-type U6 coding region and promoter elements are shown on the left and were determined as for panel B (not shown). Lys., lysate.

6 and 7) that are strongly protected from MNase cleavage on the wild-type gene. Interestingly, the T_7 stretch (positions −12 to −18) of both the wild-type and the Δ42 alleles is protected from cleavage in the cell lysate compared with that in naked DNA, independent of the level of protection over the TATA box (Fig. 7B, lanes 4 to 7). Loss of protection as well as the appearance of enhancements over the TATA box and at position −43 suggest that binding of TFIIIB has been partially disrupted or altered. Because the Δ42 mutant allele is fairly well transcribed in vivo (Fig. 6C), TFIIIB must be at least transiently associated with the *SNR6* promoter. However, the protection afforded by partial occupancy may be difficult to detect in the presence of enhanced cleavages on the copies of *SNR6* from which TFIIIB (or one of its subunits) has dissociated. DNase I analysis of *snr6-Δ42* chromatin reveals a substantial loss of protection over the TFIIIB-binding region (data not shown). Whether the continued protection of the T_7 stretch reflects continued binding of a subunit of TFIIIB or binding of a different protein is not clear (see Discussion). What is clearly shown is that a mutation over 150 bp downstream of the *SNR6* TATA box has a profound effect on protein binding at the TATA box in vivo.

To examine whether the protection from MNase digestion between the A and B blocks of *SNR6* is due to or dependent on TFIIIC binding, we performed chromatin footprinting on a strain in which *SNR6* with a lethal point mutation (T237C) in the B block has been integrated into the chromosomal *SNR6* locus (Fig. 7C) (31). The T237C mutation decreases in vivo expression of *SNR6* to just 2% of wild-type levels; furthermore, the *snr6-T237C* allele does not compete for binding of TFIIIC by *SNR6* in vitro (31). Comparison of the MNase protection pattern on the transcribed strand of *snr6-T237C* (Fig. 7C, lanes 1 and 2) and the wild-type gene (Fig. 7C, lanes 3 and 6) shows that the protection between the A and B blocks remains. However, the pattern of MNase protection over the upstream region is drastically different, with the pattern in the B block mutant allele closely resembling that of Δ42 (Fig. 7C, lane 7). It is somewhat surprising that the loss of protection over the upstream regions of the lethal B block and Δ42 mutants is so similar (Fig. 7C, compare lanes 2 and 7) given that the Δ42 mutant is much more efficiently transcribed (see Discussion). The chromatin footprinting results suggest that the protection from MNase digestion between the A and B blocks of *SNR6* is not correlated with transcription and may be established independently of the binding of TFIIIC and TFIIIB.

To further test our assumption that the MNase protection

over the upstream region of *SNR6* is due to TFIIIB binding, we examined the chromatin footprint of the TATAbox-sub allele of *SNR6* (Fig. 7C, lanes 4 and 5). Although the cleavage pattern is different in the upstream region because of the change in sequence (Fig. 7C, compare lanes 5 and 6), it is clear that this mutation alters the MNase footprint in a manner similar to Δ42, specifically with respect to enhanced cleavage at position −43 and decreased cleavage at position −4 (compare lanes 4 and 7). This result supports the notion that the effect of the Δ42 mutation is to disrupt TFIIIB binding via an as-yet-undetermined mechanism.

DISCUSSION

Role of the A block in positioning of TFIIIB on *SNR6*. Our results define a central role of the A block in initiation of *SNR6* transcription in vivo. Earlier studies demonstrated that both the TATA box and the A block are involved in *SNR6* start site selection in vivo (7, 13) but did not clearly define the quantitative and qualitative differences in their activities. Here we have used an allele of *SNR6* containing tandemly duplicated start sites to uncouple A block- from TATA box-directed initiation. We find that the A block dominates start site selection in vivo (Fig. 2 and 3). The TATA box serves primarily to modify *SNR6* A block selection (A1 versus A2 block) and to direct the precise choice of the start site in the window defined by the A block (e.g., +1 versus +5).

Chalker and Sandmeyer (7) used a different strategy to uncouple start site selection by sequences upstream and downstream of the start site and obtained an apparently different result. They deleted 12 bp between the transcription start site and the A block and found that the major in vivo transcript was 12 bp shorter than wild-type U6 RNA, consistent with initiation directed primarily by the TATA box at the wild-type start site. However, the 12-bp deletion brought the cryptic A2 block into the proper position to direct initiation at the wild-type start site, so it is likely that this element contributed to selection of the observed start site. Indeed, complete deletion of the TATA box did not abolish the use of the wild-type start site. Furthermore, because the start site region was not duplicated, sequence selectivity of Pol III may have skewed start site selection. We suggest, therefore, that these prior experiments do not permit one to draw conclusions concerning the relative contribution of the TATA box and A block to start site selection.

We presume that the *SNR6* A block acts by binding TFIIIC and promoting its interaction with TFIIIB, as does the tRNA gene A block. It is unlikely that TFIIIB interacts with the A block directly, since initiation at the A block-dependent start site of the *snr6-start-dup* allele was not detected with purified TFIIIB and Pol III (Fig. 4). We have not observed a clear A block footprint on the yeast U6 RNA gene in chromatin (Fig. 5B and C), as is also the case for some but not all yeast tRNA genes (25–27). Absence of the A block footprint may reflect a relatively low affinity of the *SNR6* A block for TFIIIC, as a consequence of its sequence and/or its distance from the downstream B block. In vitro, TFIIIB remains stably associated with the upstream region of a tRNA gene through many rounds of transcription even when TFIIIC has been stripped from the gene with heparin (33). If TFIIIB binds with similar stability in vivo on *SNR6*, then there is no need for TFIIIC to remain bound to the *SNR6* A block once TFIIIB is assembled upstream and the gene is being actively transcribed. Burnol et al. (6) found that a 3-bp substitution (called Aup) that increases the match of the *SNR6* A block to the consensus sequence increases U6 RNA synthesis in a crude yeast extract or with purified components, using a TFIIIC-dependent nucleosomal template. Indeed, the Aup mutation allows *SNR6* transcription in the absence of the downstream B block in these TFIIIC-dependent systems, as well as in vivo. It would be interesting to look for a chromatin footprint over the A block of the *snr6-Aup* allele.

Role of the upstream sequences in the positioning of TFIIIB on *SNR6*. We have shown that *SNR6* upstream sequences modify start site selection by the A block and can at least partially assume the role of the A block when the latter is mutated or when the A-to-B block spacing is altered. Since identical components of TFIIIB are known to be necessary for in vitro transcription of *SNR6* and TATA-less tRNA genes (23, 30), involvement of the *SNR6* upstream region in TFIIIB positioning is likely to be due to the combination of a weak TFIIIC-A block interaction and the presence of upstream sequences that are optimal for TFIIIB binding.

A key component of the TFIIIB binding site on *SNR6* is the TATA box, which presumably is recognized by the TBP subunit. Substitution of the TATA box results in the loss of MNase and DNase I protection over much of the upstream region of *SNR6* chromatin (Fig. 7C and data not shown). Loss of MNase protection in this region is also seen in the presence of the Δ42 and T237C mutations (Fig. 7C). One possible interpretation of the apparent lack of correlation between the extent of MNase protection over the TATA box and the transcription activity for the mutant alleles is that weak binding of TFIIIB to the *snr6-TATA-box* and *snr6-Δ42* alleles is sufficient for the observed level of in vivo expression but is disrupted by the footprinting procedure. We show that the sequence selectivity of the interaction of TFIIIB with the *SNR6* TATA box is high (Fig. 4): TFIIIC-independent binding of TFIIIB to the *SNR6* TATA box is as sensitive to point mutation as is binding of TFIID to Pol II TATA boxes (62). Furthermore, analysis of the T7-sub allele in the purified transcription system indicates that sequences downstream of the TATA box, including part but not all of a stretch of seven T residues in the nontranscribed strand, are also important for *SNR6* transcription in vitro (Fig. 4). The T7-sub mutation has only a very slight effect on its own in vivo, but it would be interesting to test it in combination with either the A1-sub or Δ42 mutations, which increase in vivo sensitivity to upstream mutations.

Intriguingly, the T_7 stretch is strongly protected from MNase digestion in the *snr6-Δ42*, *snr6-T237C*, and *snr6-TATAbox-sub* alleles (as well as in wild-type chromatin), even though the TATA box region is hypersensitive to MNase in these mutant alleles (Fig. 7C). Photocross-linking (2) and footprinting (32) studies on a tRNA gene identify the 70-kDa subunit of TFIIIB as the most likely candidate for binding to the region defined by the T7-sub mutation. The 70-kDa subunit (also called Tds4, Brf1, and Pcf4) has been proposed to be a homolog of TFIIB (4, 8, 41), which binds downstream of Pol II TATA boxes and stabilizes the DNA binding of TBP (reviewed in reference 21). However, the persistence of the T_7 stretch protection in the *snr6-T237C* allele, which is transcribed at only 2% of the wild-type level, suggests that some protein other than TFIIIB contributes to the MNase footprint in this region.

Interestingly, an eight-of-nine match to the region encompassing the T_7 stretch of *SNR6* has also been reported in an analogous position in the *S. cerevisiae RPR1* gene, which encodes the RNA subunit of nuclear RNase P (37). The promoter of this gene contains a B block that is a poor match to the consensus as well as a properly positioned A block. In addition, matches to the first 4 bp of the *SNR6* TATA box are found at bp −27 and −37 relative to the transcription initiation site. Conceivably, departure of the *RPR1* B block from the

consensus weakens the binding of TFIIIC and thus makes TFIIIB binding more dependent on upstream sequences.

Positional constraints on B block function. A surprising finding of our mutational analysis is that two mutations, TATA box-sub and Δ42, which are over 150 bp apart and each of which has only a relatively minor effect on the level of expression of *SNR6* in vivo (13), almost abolish U6 RNA synthesis when present in combination (Fig. 6). The synergism observed in the double mutant must reflect cooperative interactions between the upstream and downstream regions, most likely mediated by TFIIIB and TFIIIC. Physical evidence for this cooperative interaction came from the chromatin footprinting experiments, which revealed a dramatic increase in the MNase sensitivity of the *SNR6* TATA box upon introduction of the Δ42 deletion (Fig. 7B). The effect of deletion of 42 bp between the terminator and the B block argues against the model that the B block acts as an enhancer element (6). Rather, it implies that productive binding of TFIIIC to *SNR6* is sensitive to A-to-B block spacing, since the identity of the DNA sequence in this region is likely to be unimportant (20). Given the range of A-to-B block spacings allowed in tRNA genes, this result was unexpected and implies the existence of structured chromatin between the *SNR6* A and B blocks. We have previously shown that deletion of 84 bp of this downstream region has less of an effect on transcription than deletion of either half of the 84 bp sequence (the Δ42 mutation used here corresponds to the upstream half of the 84 bp region [13]). Since 84 bp represents one turn around a nucleosome, the different effects of 42- and 84-bp deletions might be taken as a reflection of nucleosomal packaging of *SNR6*.

Role of chromatin structure in *SNR6* expression. Some of us have proposed that a positioned nucleosome brings the *SNR6* A and B block elements closer together, facilitating the binding of TFIIIC (13). There are now several examples of genes on which positioned nucleosomes are thought to facilitate transcription (reviewed in references 54 and 63). There is evidence for positioned nucleosomes on the *Drosophila hsp26* (55) and alcohol dehydrogenase (29) genes, as well as on the *Xenopus* vitellogenin B1 gene (49). In each case, the nucleosomes are proposed to juxtapose distant regulatory elements and enhance their recognition by *trans*-acting factors.

The MNase digestion pattern obtained with *SNR6* chromatin, which includes a large region of protection between the terminator and the B block (Fig. 7), is consistent with previously published patterns interpreted as reflecting nucleosomal packaging (17, 29, 47). The similarity of the footprint in this region in the wild-type allele and an allele that is very poorly transcribed (*snr6-T237C*) indicates that the chromatin structure is established independently of an active transcription complex. Thus, the protection is unlikely to be due to binding of either TFIIIB or TFIIIC. The fact that the region of continuous protection from MNase is only 90 to 100 bp in length rather than the 146 bp expected for a nucleosome could be explained by partial displacement or disruption of the nucleosome in the U6 RNA coding region. Such displacement could be mediated by derepressing proteins, such as the SWI/SNF complex (10, 28 [and references therein]). Nevertheless, it is also possible that the MNase protection of *SNR6* chromatin is due to binding of nonhistone proteins. These could be either general chromatin proteins or as-yet-unidentified Pol III-specific factors. In any case, the results presented here suggest that the *S. cerevisiae* U6 RNA gene may be another example of a gene in which chromatin structure plays a positive role in assembly of transcription complexes.

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