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RNA 2000 6: 1859-1869

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Disruption of the 5′ stem-loop of yeast U6 RNA induces trimethylguanosine capping of this RNA polymerase III transcript in vivo

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ABSTRACT

Primary transcripts made by RNA polymerase II (Pol II), but not Pol I or Pol III, are modified by addition of a 7-methylguanosine (m^7G) residue to the triphosphate 5′ end shortly after it emerges from the polymerase. The m^7G "caps" of small nuclear and small nucleolar RNAs, but not messenger RNAs, are subsequently hypermethylated to a 2,2,7-trimethylguanosine (TMG) residue. U6 RNA, the only small nuclear RNA synthesized by Pol III in most eukaryotes, does not receive a methylguanosine cap. However, human U6 RNA is *O*-methylated on the 5′-terminal (γ) phosphate by an enzyme that recognizes the 5′ stem-loop of U6. Here we show that variant yeast U6 RNAs truncated or substituted within the 5′ stem-loop are TMG capped in vivo. Accumulation of the most efficiently TMG-capped U6 RNA variant is strongly inhibited by a conditional mutation in the largest subunit of Pol III, confirming that it is indeed synthesized by Pol III. Thus, methylguanosine capping and cap hypermethylation are not exclusive to Pol II transcripts in yeast. We propose that TMG capping of variant U6 RNAs occurs posttranscriptionally due to exposure of the 5′ triphosphate by disruption of protein binding and/or γ-methyl phosphate capping. 5′ truncation and TMG capping of U6 RNA does not appear to affect its normal function in splicing, suggesting that assembly and action of the spliceosome is not very sensitive to the 5′ end structure of U6 RNA.

Keywords: A block; Pol III; snRNA; splicing; TATA box; TMG

INTRODUCTION

Eukaryotes have three nuclear DNA-dependent RNA polymerases, designated Pol I, Pol II, and Pol III, which synthesize different classes of RNA. The primary transcripts made by all three RNA polymerases have a triphosphate at their 5′ end, but the triphosphate is often removed or modified by RNA processing enzymes. For example, Pol I synthesizes the precursor ribosomal RNA (rRNA), which undergoes multiple exo- and endonucleolytic cleavages to form the mature rRNAs with monophosphate 5′ termini. In contrast, RNAs synthesized by Pol II, including messenger RNA (mRNA) and most small nuclear and nucleolar RNAs (snRNA and snoRNA), often retain the 5′ end of the primary transcript. However, the 5′-triphosphate of Pol II transcripts is modified by several enzymatic activities, the net result of which is attachment of a 7-methylguanosine (m^7G) residue to the γ-phosphate through a 5′-phosphoester linkage (reviewed in Shuman, 1995). The m^7G "cap" is added shortly after initiation of transcription by enzymes that are associated with the C-terminal domain of the largest subunit of Pol II, so methylguanosine capping is generally presumed to be Pol II specific (reviewed in Shuman, 1997).

The m^7G caps of snRNAs and snoRNAs, but not mRNAs, are subsequently hypermethylated to a 2,2,7-trimethylguanosine (TMG) nucleoside. In vertebrates, snRNA cap hypermethylation appears to occur in the cytoplasm, through which the snRNAs transit during their maturation and packaging into snRNPs (Mattaj, 1986; Plessel et al., 1994). Hypermethylation guides the reimport of snRNPs into the nucleus (Fischer & Lührmann, 1990; Hamm et al., 1990; Huber et al., 1998). In contrast, vertebrate snoRNAs do not enter the cytoplasm, but rather appear to be hypermethylated by a nuclear enzyme (Terns & Dahlberg, 1994; Terns et al., 1995). Methylguanosine caps of yeast snRNAs and snoRNAs are also hypermethylated (Wise et al., 1983; Riedel et al., 1986), but the subcellular compartment in which hypermethylation occurs is not known.

Pol III transcripts exhibit a variety of 5′ end structures. Transfer RNAs (tRNAs), like rRNAs, are nucleolytically processed and have a monophosphate 5′ end.

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5S rRNA, the only rRNA synthesized by Pol III rather than Pol I, retains the triphosphate terminus of the primary transcript in an uncapped form. U6 RNA, the only snRNA synthesized by Pol III in most eukaryotes studied, also retains the 5′ triphosphate, but is *O*-methylated on the terminal (γ) phosphate, at least in humans (Singh & Reddy, 1989). Synthesis of the γ-methyl phosphate cap of U6 RNA in human cells is dependent upon structural determinants at the base of a conserved 5′-terminal stem (Singh et al., 1990). Indeed, yeast U6 RNA is γ-methylated in human cell extracts. However, it is not known whether U6 RNA receives a γ-methyl phosphate cap in the yeast cell.

Yeast (*Saccharomyces cerevisiae*) U6 RNA is synthesized by Pol III from the *SNR6* gene (Brow & Guthrie, 1988; Moenne et al., 1990). The in vivo transcription start site of *SNR6* is specified by at least two promoter elements, an upstream TATA box and an intragenic A block (Eschenlauer et al., 1993; Gerlach et al., 1995). Certain mutations in these elements result in synthesis of a subset of transcripts that are either elongated or truncated at their 5′ ends relative to wild-type U6. Here we show that, unexpectedly, variant forms of yeast U6 RNA produced from these mutant alleles are TMG capped in vivo, as judged by immunoprecipitation with a TMG-specific antibody. The efficiency of capping varies considerably among the different variant forms, but all capped species appear to be efficiently hypermethylated as judged by much lower reactivity with an antibody specific for the m^7G cap. We developed a gel-shift assay to screen a collection of mutant U6 RNAs for TMG capping, and find a good correlation between the efficiency of TMG capping and the extent of disruption of the base of the 5′ stem. Experiments using the most efficiently capped variant show that disruption of the 5′ stem does not simply increase antibody access to a pre-existing TMG cap, and confirm that the capped transcripts are indeed synthesized by Pol III. We propose that posttranscriptional TMG capping occurs by default in the yeast nucleus on U6 molecules with a 5′ triphosphate that is not blocked by γ-methylation or protein binding, and suggest that default capping and hypermethylation of nuclear RNAs with unblocked triphosphate termini may be a general phenomenon not restricted to Pol II transcripts.

RESULTS

Immunoprecipitation of variant U6 RNAs with antibody against the TMG cap

The effects of mutations in the yeast U6 RNA gene are scored in a strain in which the chromosomal *SNR6* locus is deleted and the *SNR6* allele to be tested is provided on a centromere plasmid. All of the alleles described below support viability of the *SNR6* deletion strain in the absence of a wild-type *SNR6* plasmid and thus produce adequate levels of functional U6 RNA, which is essential for pre-mRNA splicing. A consensus TATA box element upstream of *SNR6* contributes to transcription start site selection in vivo. Severe mutations within the TATA box result in truncated 5′ ends (Fig. 1; Eschenlauer et al., 1993). Complete substitution of the 8-bp TATA box (TATAbox-sub) produces in vivo transcripts with 5′ ends at positions +1, +5, +7, and +12 relative to the wild-type +1 5′ end, whereas substitution of only the first 4 bp of the TATA box (TATA-sub) results in +1 and +5 transcripts. An intragenic "A block" promoter element also influences start site selection. A 3-bp substitution in the A block (A1-sub) results in transcripts with 5′ ends at positions −4, −2, +1, and +12, whereas a similar mutation in an overlapping cryptic A block (A2-sub) has no effect on start site selection (Fig. 1; Eschenlauer et al., 1993).

To examine the cap structure of the *SNR6* transcripts produced in vivo, total cellular RNA from wild-type and mutant strains was incubated with Protein A-Sepharose-bound polyclonal antibody specific for the 2,2,7-trimethylguanosine cap (Lührmann et al., 1982), and immune complexes were precipitated. U6 RNA present in the supernatant and pellet fractions was detected by primer extension of a ^{32}P-labeled oligonucleotide complementary to the 3′ end of U6. As expected, wild-type U6 RNA shows little or no cross-reactivity with the anti-

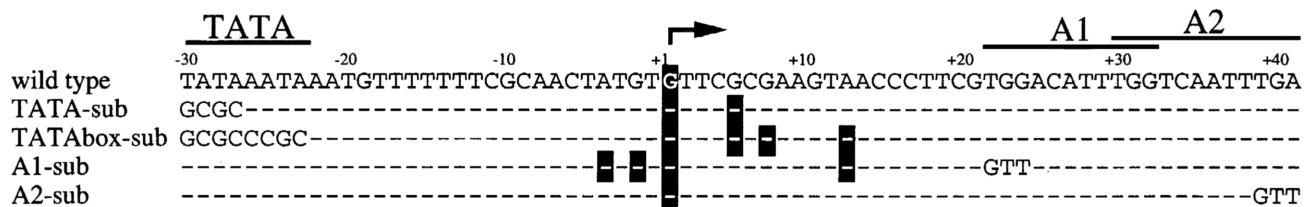

FIGURE 1. *SNR6* promoter mutations that affect transcription start site selection. The sequence of wild-type *SNR6* from position −30 to +40 is shown in the top line. The location of the wild-type transcription start site (+1) is marked with an arrow. The locations of the TATA box, the dominant A block (A1) and a cryptic, overlapping A block (A2) are indicated by bars. Substitutions in the indicated mutant alleles are shown below the wild-type *SNR6*; dashes denote wild-type sequence. The observed transcription start sites are highlighted in black.

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TMG antibody under conditions that result in nearly complete precipitation of the TMG-capped U4 snRNA, a Pol II transcript (Fig. 2A, lanes 1 and 2).

Strikingly, the variant transcripts produced in vivo from both the TATAbox-sub and A1-sub alleles are bound by anti-TMG antibody, with varying efficiency. The TATAbox-sub transcripts with a 5′ end at +12 are efficiently precipitated whereas normal length (+1) transcripts are not (Fig. 2A, lanes 9 and 10). Transcripts with +5 and +7 5′ ends are precipitated with intermediate efficiency. Increasing the amount of total cellular RNA twofold or decreasing it fourfold does not alter the fractional distribution of each TATAbox-sub transcript between the pellet and supernatant (data not shown), which is therefore not due to competition of the TATAbox-sub transcripts for limiting antibody. Furthermore, the +5 transcript produced from the TATA-sub allele (Fig. 2A, lanes 7 and 8) is precipitated with approximately the same efficiency as the +5 transcript from the TATAbox-sub allele, indicating that the low anti-TMG cross-reactivity is an intrinsic property of the +5 transcript. Normal rabbit serum does not precipitate any of the TATAbox-sub transcripts or the U4 RNA (Fig. 2A, lanes 11 and 12).

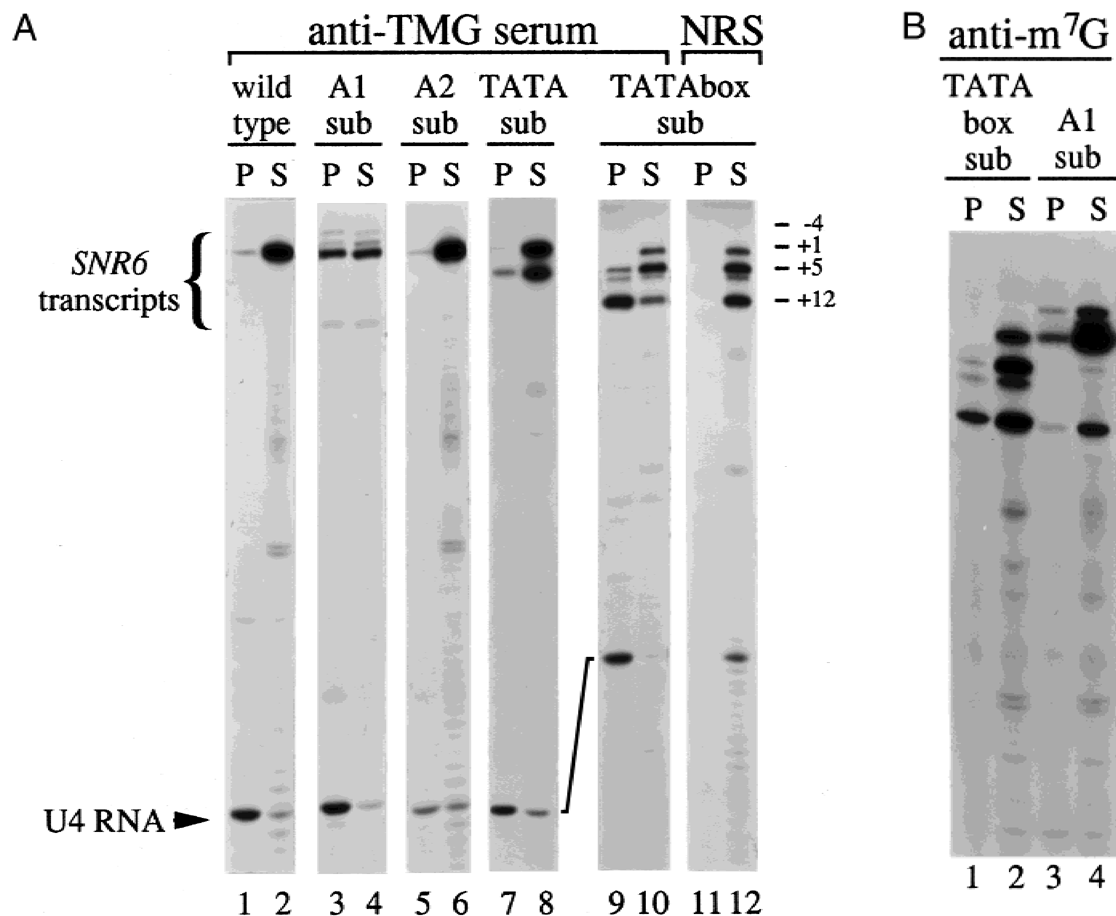

FIGURE 2. Immunoprecipitation of in vivo transcripts from mutant *SNR6* alleles with anti-2,2,7-trimethylguanosine (TMG) (**A**) and anti-7-methylguanosine (m^7G) IgG (**B**). Pellet (P) lanes contain RNA that bound to the indicated IgG linked to Protein A-Sepharose; Supernatant (S) lanes contain RNA that did not bind. NRS is a control utilizing normal rabbit serum. The *SNR6* transcripts and endogenous U4 RNA (in **A**) were detected by primer extension analysis; the positions of their cDNAs are indicated in **A**. U4 RNA has a TMG cap and serves as a control for reactivity of the anti-TMG IgG (Riedel et al., 1986; Siliciano et al., 1987). **C**: 5′ stem-loop structure of *S. cerevisiae* U6 RNA (Fortner et al., 1994). The γ-monomethyl phosphate 5′-cap found in vertebrates, but not known to exist in yeast, is shown parenthetically. The variant initiation sites (+5, +7, +12) caused by the TATAbox-sub mutation are shown, as are the substitutions present in A1-sub mutant transcripts.

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The A1-sub transcripts are all precipitated with approximately 50% efficiency by the anti-TMG antibody, including the +1 transcript (Fig. 2A, lanes 3 and 4). Therefore, the length of the transcript per se does not correlate with anti-TMG reactivity. Unlike the TATAbox-sub +1 transcript, which is wild type in sequence, the A1-sub transcripts all have a 3-nt substitution in the 5′ stem (see Fig. 2C). The A2-sub triple substitution, which falls downstream of the 5′ stem (Figs. 1 and 2C), does not alter the transcription start site and does not confer anti-TMG cross-reactivity to the transcript (Fig. 2A, lanes 5 and 6). Therefore, it appears that mutations that alter the structure of the 5′ stem, either by truncation or substitution (see Fig. 2C), result in at least partial TMG capping of U6 RNA.

Cross-reaction of the anti-TMG IgG with some other modified base is unlikely because of the high degree of specificity of the antibodies (Lührmann et al., 1982) and because the same variant transcripts are also precipitated, in smaller amounts but the same proportions, with polyclonal antiserum specific for m^7G cap (Munns et al., 1982), a precursor of the TMG cap (Fig. 2B). Because the anti-TMG and anti-m^7G antibodies have nonoverlapping specificities (Neuman de Vegvar & Dahlberg, 1990; Terns & Dahlberg, 1994; Terns et al., 1995), total methylguanosine capping of a given transcript is measured as the sum of the pelleted transcript in the two immunoprecipitation assays (compare Figs. 2A and 2B), and the efficiency of hypermethylation is indicated by the difference. We conclude that most, if not all, of the +12 transcript of the TATAbox-sub allele is methylguanosine capped, and hypermethylation of the +12 transcript is estimated to be 80–90% efficient. Other variant transcripts are less efficiently capped but, when capped, are mostly hypermethylated.

A gel-shift assay for TMG capped RNAs

We could not rule out the possibility that immunoprecipitation of variant forms of U6 RNA with anti-TMG antibody was due to their specific association with some other RNA that bears a TMG cap. Heating the RNA solution to 90 °C to disrupt any base-pairing interactions and quick chilling on ice to prevent reannealing before addition of antibodies did not alter the precipitation pattern (data not shown). However, association of the variant transcripts with a TMG-capped RNA could conceivably occur during the subsequent 4 °C incubation with antibody, even though no association of wild-type U6 with U4 RNA was observed despite their extensive complementarity (Brow & Guthrie, 1988). An attempt to detect the TMG capped *SNR6* transcripts by anti-TMG antibody probing of a Northern blot of total cellular RNA fractionated on a denaturing gel (Midwestern blotting; Rasmussen & Culbertson, 1996) was unsuccessful (T. Rasmussen and M. Culbertson, pers. comm.), presumably because of the relatively low abundance of the variant transcripts.

We therefore developed an alternative assay that can simultaneously detect RNA–RNA and RNA–antibody interactions. Specific snRNAs present in total cellular RNA were labeled by hybridization in solution with a ^{32}P-labeled complementary oligonucleotide. The hybrids were then resolved on a native gel, as done previously (Li & Brow, 1993). RNA–RNA interactions, such as U4/U6 pairing, can be detected on the nondenaturing gel because of decreased mobility of the complex relative to the free RNAs. To detect the presence of a TMG cap, anti-TMG IgG was incubated with the oligonucleotide-RNA hybrids before electrophoresis. Binding of IgG to RNA was detected as a discrete decrease in the gel mobility of the hybrid. The assay worked well with a high-titer anti-TMG monoclonal antibody, H20 (Bochnig et al., 1987; the H20 antibody also recognizes 7-methylguanosine cap). A commercially available anti-TMG monoclonal antibody (K121 from Oncogene Research Products, Cambridge, Massachusetts) also worked well. The assay did not work well in our hands with the TMG-specific polyclonal antibody used in our initial immunoprecipitation experiments.

The gel-shift assay was first validated by examining U4 RNA. Lanes 1 and 2 of Figure 3 show total cellular RNA from a wild-type strain incubated with ^{32}P-labeled oligonucleotides complementary to U4 and U6 RNAs, respectively, after heating to 90 °C to dissociate any existing U4/U6 complex. In each of the remaining lanes, both U4 and U6 oligonucleotides were added. Incubation of the RNA–oligo hybrids with as little as 0.1 μg of the anti-TMG monoclonal antibody (mAb) before electrophoresis resulted in a shift of most of the U4 RNA to a position much closer to the origin (Fig. 3, lane 4). In contrast, the mobility of U6 RNA was not shifted, even with much higher amounts of anti-TMG mAb (Fig. 3, lanes 4–6, and data not shown).

We next confirmed that the gel-shift assay can detect caps on the variant *SNR6* transcripts that were previously shown to precipitate with anti-TMG antibody. In the experiment shown in lanes 7–12 of Figure 3, only the U6 RNA oligonucleotide was added, but other experiments with each RNA preparation established that U4 RNA could be efficiently shifted with the concentration of H20 mAb used (data not shown). As observed in the previous experiment, little or no wild-type U6 RNA is shifted by the H20 mAb (Fig. 3, lanes 7 and 8). In contrast, the fastest migrating TATAbox-sub transcripts, which correspond to +12 RNA (Li & Brow, 1993), are efficiently shifted (Fig. 3, lanes 9 and 10). A significant fraction of the A1-sub transcripts is also shifted by the H20 mAb (Fig. 3, lanes 11 and 12).

These results prove that the anti-TMG antibody binds directly to variant forms of U6 RNA. When incubated in the absence of antibody, the variant transcripts migrate with wild-type U6 RNA, indicating that they are not com-

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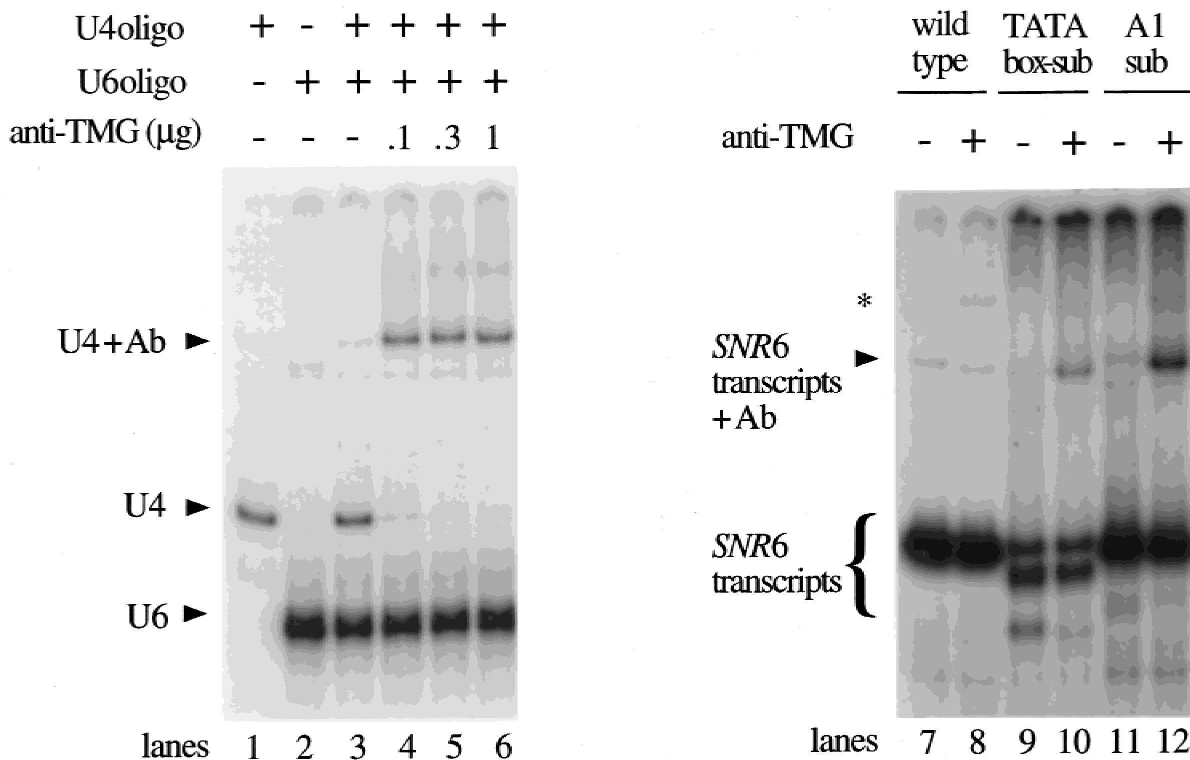

FIGURE 3. Gel-shift assay for TMG capped snRNAs. Total cellular RNA from wild-type (lanes 1–8), TATAbox-sub (lanes 9 and 10), or A1-sub (lanes 11 and 12) yeast strains was heated at 90 °C for 1 min to disrupt any U4/U6 RNA complex, and then incubated at 37 °C for 15 min with ^{32}P-labeled oligonucleotide complementary to U4 RNA (U4B; lanes 1, 3–6) and U6 RNA (U6D; lanes 2–12). H20 mAb was added as indicated (−: no mAb; +: 1 μg mAb) and the incubation was continued at 4 °C for 1 h. Hybrids were then resolved on a native 9% polyacrylamide gel. The positions of free RNAs and antibody-RNA complexes is indicated on the left of each panel. The asterisk indicates the position in lane 8 of a small amount of U4/U6 RNA complex that is shifted by the H20 mAb.

plexed with another RNA. The fraction of variant *SNR6* transcripts bound by anti-TMG antibody in the gel-shift assay is consistently lower than in the immunoprecipitation assay. For this reason, the gel-shift assay may not be ideal for precisely quantifying the fraction of TMG capped transcript. Nevertheless, the gel-shift assay is a sensitive indicator of capping and can additionally detect association of the transcript being probed with other RNAs. Furthermore, it is a very rapid and convenient method, which facilitates the screening of large numbers of RNA samples.

TMG capping of SNR6 transcripts is not due to polymerase switching, but correlates with disruption of the base of the 5′ stem

Having established that the anti-TMG antibody binds directly to the variant *SNR6* transcripts, we next addressed the mechanism of TMG capping of U6 RNA. It is generally assumed that methylguanosine capping is obligatorily cotranscriptional and restricted to Pol II transcripts (see Shuman, 1997). Therefore, a potential explanation for TMG capping of the variant *SNR6* transcripts is switching from Pol III to Pol II as a result of mutations in the *SNR6* promoter elements. Such polymerase switching has been observed for vertebrate U6 RNA genes as a result of mutations in the TATA box (Mattaj et al., 1988; Lobo & Hernandez, 1989). However, this explanation is highly unlikely in the case of the yeast U6 RNA gene for a number of reasons. First, the promoter structure of the yeast U6 gene is very similar to that of canonical Pol III transcription units and very different from the promoters of yeast genes transcribed by Pol II. In contrast, vertebrate U6 gene promoters differ from promoters of Pol II-transcribed snRNA genes only by the presence of a TATA box in the former (reviewed in Dahlberg & Lund, 1991; Kunkel, 1991; Hernandez, 1992; Willis, 1993). Second, synthesis of the efficiently capped +12 transcript from the TATAbox-sub allele requires both the A block promoter element (Eschenlauer et al., 1993) and downstream sequences encompassing the B block element (Gerlach et al., 1995; L. Loos and D.A. Brow, unpubl.), indicating that the Pol III-specific initiation factor TFIIIC is required. Third, the variant *SNR6* transcripts efficiently terminate at the Pol III termination signal (T_{10}), as evidenced by their size (Figs. 3 and 4, and data not shown). No read-through transcripts were detected from either the TATAbox-sub

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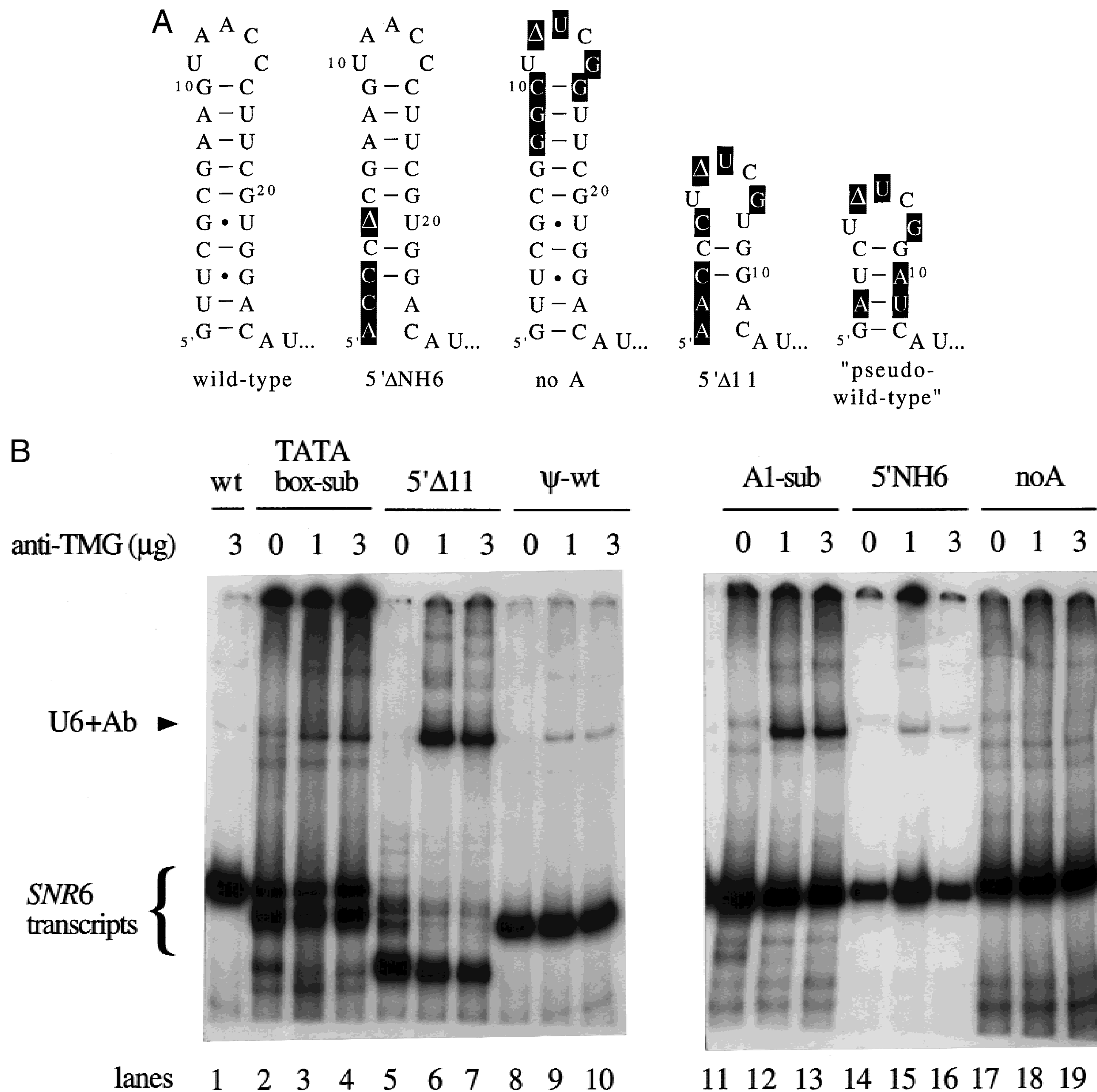

FIGURE 4. Effect of 5′ stem structure on TMG capping of U6 RNA. **A**: Proposed secondary structure of the 5′ stem regions of wild-type and mutant U6 RNAs. Substitutions are highlighted. **B**: Gel-shift cap analysis of mutant U6 RNAs. Total cellular RNA from strains containing the indicated *SNR6* allele was incubated with ^{32}P-labeled oligonucleotide complementary to U6 RNA (U6D) and 0, 1, or 3 μg H20 anti-TMG mAb. The positions of free RNAs and RNA-mAb complexes is indicated on the left.

or A1-sub alleles when using downstream primers in a primer extension assay. Therefore, methylguanosine capping of variant U6 RNAs is most likely due to post-transcriptional modification of a Pol III product, and not due to polymerase switching.

A hypothesis that could explain selective posttranscriptional capping of variant, but not wild-type, U6 RNA is that the 5′ triphosphate of wild-type U6 RNA is blocked by binding of a protein to the intact 5′ stem and/or by γ-methylphosphate capping in a 5′ stem-dependent fashion. Disruption of the 5′ stem therefore exposes the 5′ triphosphate of U6 to methylguanosine capping enzymes present in the nucleus. A strong prediction of this model is that mutations that alter the 5′ stem of U6 RNA without altering any promoter elements would also result in methylguanosine capping. In particular, +12 U6 RNA should be efficiently capped even in the absence of mutations in the TATA box or A block. To test this prediction, we assessed the extent of in vivo capping of several mutant U6 RNAs, using the gel-shift

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assay (Fig. 4). We created a mutant allele in which the first 11 bp of the U6 RNA-coding region are deleted (5′Δ11). The intact TATA box of the 5′Δ11 allele should collaborate with the A2 block (Fig. 1) to direct initiation at position +1 of this allele, which is equivalent to +12 of wild-type *SNR6*. An *SNR6* disruption strain containing only the 5′Δ11 allele is viable, and primer extension analysis of total RNA from the 5′Δ11 strain detects the +1 transcript as the major product (75% of total U6 RNA; data not shown). The remaining transcripts include 4–10 nt of upstream sequence and are also not expected to form a stable 5′ stem. Therefore, an intact 5′ stem is not required for yeast U6 RNA function.

As predicted by the posttranscriptional capping model and contrary to the polymerase-switching model, the transcripts of the 5′Δ11 allele are efficiently capped (Fig. 4B, lanes 5–7). An antibody-shifted complex is detected that is identical in mobility, but of greater yield, than that from TATAbox-sub RNA (Fig. 4B, lanes 2–4). Consistent with the gel-shift results, two-thirds of the 5′Δ11 +1 transcript is immunoprecipitated with the H20 mAb, as is three-fourths of the minor, longer 5′Δ11 transcripts (data not shown). We also tested "pseudo-wild-type" U6 RNA (Madhani et al., 1990), which is 12 nt shorter than wild-type U6 RNA due to replacement of the *S. cerevisiae* 5′ stem with the *Schizosaccharomyces pombe* 5′ stem (Fig. 4A). The pseudo-wild-type transcript is similar in size to the 5′Δ11 transcript, but can form a stable 5′ stem, which likely explains its slower mobility in the native gel (Fig. 4B, lanes 8–10). The pseudo-wild-type transcript is inefficiently capped, consistent with its intact, albeit variant, 5′ stem.

Another viable deletion construct, 5′ΔNH6, was also tested. This deletion replaces all *SNR6* DNA upstream of position +6 with vector DNA, but fortuitously contains a properly positioned TATA-like sequence and so is efficiently transcribed (Brow & Guthrie, 1990). The 5′ΔNH6 transcript is only 1 nt shorter than wild-type U6, but differs in sequence at the base of the 5′ stem (Fig. 4A). This transcript is methylguanosine capped with low efficiency (Fig. 4B, lanes 14–16). To assess the contribution of the loop of the 5′ stem-loop to capping efficiency, we tested the "no-A" mutant, which has a loop sequence similar to that of pseudo-wild-type U6 (Fig. 4A). No capping of the no-A mutant was detected (Fig. 4B, lanes 17–19). We conclude that methylguanosine capping of *SNR6* transcripts correlates with the extent of disruption of the 5′-terminal stem of U6 RNA.

We could not rule out the unlikely possibility that all species of yeast U6 RNA (including wild-type) are TMG capped and disruption of the 5′ stem simply allows the anti-TMG antibody access to the cap. To test this possibility, we modified the gel-shift assay to use an oligonucleotide probe that must disrupt the 5′ stem to bind to U6 RNA. This oligonucleotide, called U6H, is complementary to positions 13–30 of U6 RNA, so 10 of its 18 nt will pair with the 3′ strand of the 5′ stem (see Fig. 2C). Binding of the U6H probe to wild-type U6 RNA therefore yields a hybrid in which nt 1–12 of U6 are unpaired, so that any cap present should be accessible to antibody. As expected, U6H binds more efficiently to the 5′Δ11 transcripts than to wild-type U6 RNA (Fig. 5, compare lanes 1 and 3), because it does not compete with an intramolecular structure in the 5′Δ11 transcripts. Importantly, the oligo does not inhibit binding of H20 mAb to the 5′Δ11 transcripts, even though it pairs to within 1 nt of the capped 5′ end of the major transcript. In contrast, no binding of H20 mAb to wild-type U6 RNA is observed when the U6H oligo is bound (Fig. 5, compare lanes 3 and 4). We conclude that the inability of anti-TMG antibody to bind wild-type U6 RNA is not due to masking of a TMG cap by the 5′ stem, but rather reflects the absence of a TMG cap on wild-type U6 RNA. Therefore, disruption of the U6 RNA 5′ stem must elicit TMG capping in vivo.

To confirm that the most efficiently capped form of variant U6 RNA is indeed synthesized by Pol III, we expressed the 5′Δ11 construct in cells bearing a temperature-sensitive mutation in *RPC160*, which encodes the largest subunit of Pol III (Dieci et al., 1995). Cells were grown at the permissive temperature of 25 °C, the culture was split in half, and one half was incubated

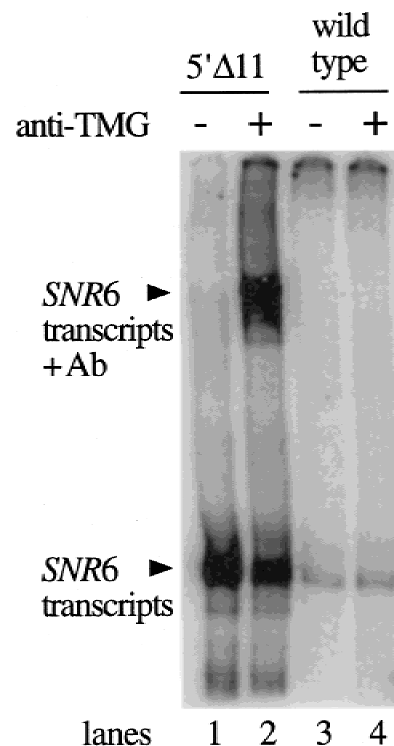

FIGURE 5. Disruption of the 5′ stem with oligonucleotide U6H does not alter the reactivity of U6 RNA with anti-TMG antibody. The gel-shift assay described in Figure 3 was performed on total RNA from strains bearing the wild-type or 5′Δ11 *SNR6* alleles, except that U6H was used as the probe oligonucleotide. U6H is complementary to the 3′ strand of the 5′ stem, and so binds less efficiently to wild-type U6 RNA than to the 5′Δ11 transcripts.

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at the restrictive temperature of 37 °C for 19 h while the other half was left at 25 °C. Figure 6A shows an analysis of the amount of wild-type and 5′Δ11 U6 RNA present in total cellular RNA extracted from the two cultures. As a control, the steady-state level of two Pol II transcripts, U4 and U5 RNAs, was also determined. Whereas the U4 and U5 RNA levels remain relatively constant after temperature shift, both wild-type and 5′Δ11 U6 RNA are severely depleted during growth at the restrictive temperature. Relative to U5 RNA, wild-type and 5′Δ11 U6 RNA levels dropped 85–90%, whereas the U4 RNA level decreased less than 20% (Fig. 6B). These results confirm that the efficiently TMG-capped 5′Δ11 U6 RNA is synthesized by Pol III.

DISCUSSION

Here we report the unprecedented observation of TMG capped forms of U6 RNA, a Pol III transcript. The polyclonal anti-m^7G and anti-TMG antibodies we have used to identify capped U6 species have been extensively characterized and their specificities are well established, and the gel-shift assay proves that the antibodies bind directly to the variant U6 RNA molecules. A number of lines of evidence indicate that methylguanosine capping of yeast U6 RNA is not due to polymerase switching, but rather is due to disruption of the 5′ stem. The structural alterations that induce methylguanosine capping of U6 RNA do not block its essential function in splicing, as forms of U6 that are efficiently TMG capped nevertheless support normal cell growth.

Because the enzymes responsible for methylguanosine capping are associated with the C-terminal domain (CTD) of Pol II in both mammals and yeast (Cho et al., 1998, and references therein), and artificial mRNAs synthesized by Pol III in mammalian cells are not capped (Gunnery & Mathews, 1995), it might be assumed that methylguanosine capping is obligatorily coupled to synthesis by Pol II. Evidence to the contrary is provided by the finding that *Trypanosoma brucei* U2 RNA is synthesized by a Pol III-like polymerase, and yet bears a TMG cap (Fantoni et al., 1994). However, it is not known whether methylguanosine capping enzymes associate with the Pol II CTD in *T. brucei*, which

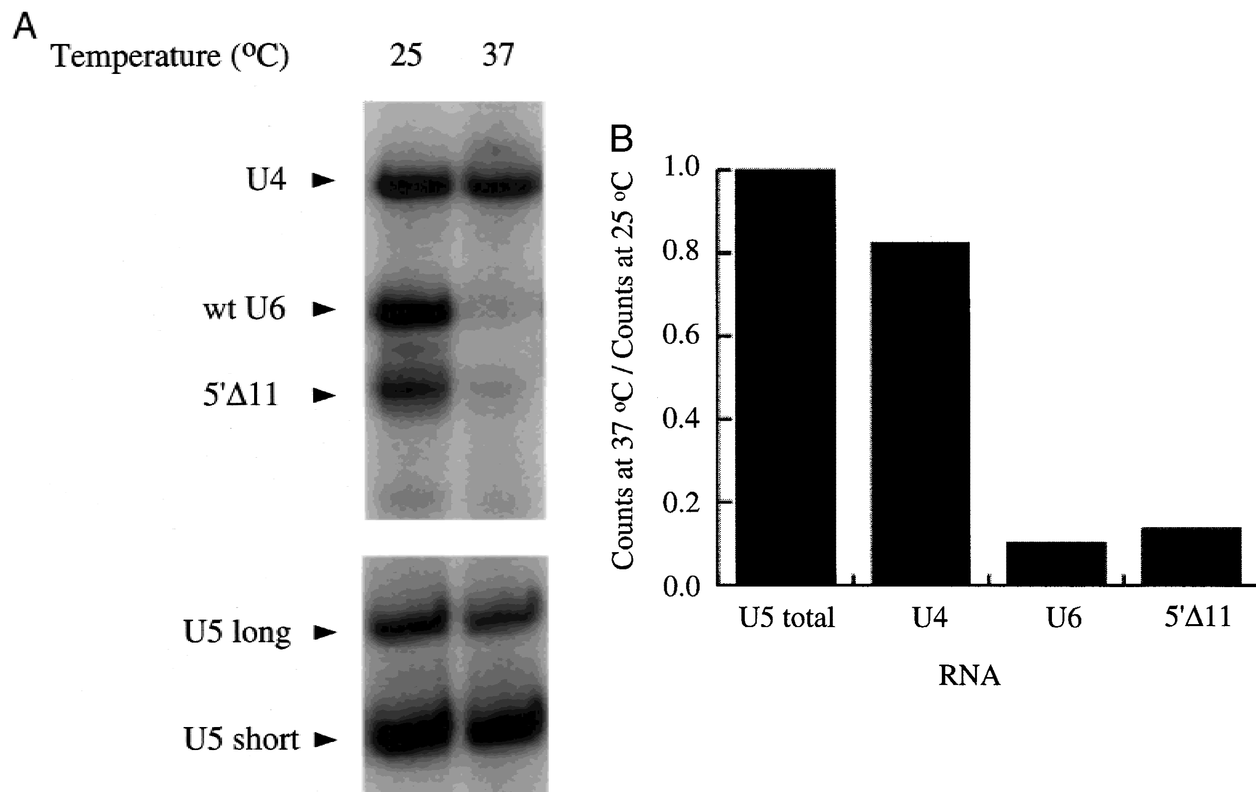

FIGURE 6. Wild-type and 5′Δ11 U6 RNAs are specifically depleted in a Pol III mutant strain grown at restrictive temperature. **A**: Total cellular RNA extracted from a strain containing a temperature-sensitive mutation in the largest subunit of Pol III, grown for 19 h at permissive (25 °C) or restrictive (37 °C) temperature, was heated at 90 °C for 1 min, then hybridized in solution to ^{32}P-labeled oligonucleotides complementary to U4, U5, and U6 RNAs. Hybrids were resolved by electrophoresis on a 6% acrylamide nondenaturing gel. Note that yeast U5 RNA is processed into short and long forms. **B**: Signals from the gel shown in **A** were quantified with a PhosphorImager SI (Molecular Dynamics). The ratio of the signals at 37 °C and 25 °C was calculated and normalized to the ratio for U5 RNA (sum of long and short forms).

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lacks the consensus heptapeptide repeats found in the yeast and human Pol II CTDs (Evers et al., 1989; Smith et al., 1989). Our results provide the first evidence that 5′ methylguanosine capping in yeast can occur on transcripts synthesized by Pol III.

It is possible that variant U6 RNAs are methylguanosine capped by enzymes distinct from those that cap Pol II transcripts. Recently, the yeast *CTL1* gene was shown to encode a RNA 5′-triphosphatase distinct from Cet1, the RNA 5′-triphosphatase responsible for the first step of mRNA capping (Rodriguez et al., 1999). Unlike Cet1, the Ctl1 protein is not essential for cell viability, and Ctl1 exhibits no genetic or physical interactions with the mRNA capping enzymes. However, we have found that in vivo capping of the 5′Δ11 *SNR6* transcripts does not require Ctl1 (data not shown). Therefore, the mRNA capping enzymes are currently the best candidates for capping of variant U6 RNAs. Perhaps the mRNA capping enzymes are present in excess of Pol II and so are not restricted to capping of nascent Pol II transcripts.

Not only are variant *SNR6* transcripts methylguanosine capped, these caps are then efficiently hypermethylated, as judged by differential reactivity with anti-m^7G and anti-TMG antibodies. Hypermethylation of vertebrate snRNA caps normally occurs in the cytoplasm and is dependent on Sm protein binding (Mattaj, 1986; Plessel et al., 1994), whereas hypermethylation of vertebrate snoRNA caps occurs in the nucleus and is dependent on the presence of specific RNA sequence elements (Terns & Dahlberg, 1994; Terns et al., 1995). It has been shown that U1 and U2 snRNAs can be hypermethylated in *Xenopus* oocyte nuclei if bound to the Sm proteins before injection (Terns et al., 1995) or if allowed sufficient time to bind endogenous proteins (Yu et al., 1998), indicating that the nuclear hypermethylase can act on snRNAs in an Sm-dependent fashion. However, U6 RNA that was m^7G capped in vitro was not hypermethylated when injected into the nucleus (Terns et al., 1995).

We propose that variant yeast U6 RNAs that receive a m^7G cap in vivo are hypermethylated by a nuclear enzyme, because U6 RNA apparently does not enter the cytoplasm (Hamm & Mattaj, 1989). Such hypermethylation could be Sm-independent, dependent on the Sm-like proteins that bind U6 RNA (Cooper et al., 1995; Séraphin, 1995; Pannone et al., 1998, Mayes et al., 1999), or dependent upon binding of U6 RNA to U4 RNA, which is bound to the Sm proteins. Failure of m^7G-capped U6 RNA to be hypermethylated in *Xenopus* nuclei (Terns et al., 1995) could be due to steric hindrance from proteins binding the intact 5′-stem. That is, disruption of the U6 5′ stem may be required both for methylguanosine capping and for hypermethylation of that cap in vivo.

If methylguanosine capping of variant U6 RNAs is a default process, then other Pol III primary transcripts may acquire methylguanosine caps. In many cases (e.g., tRNAs, RNase P RNA), the 5′ ends of Pol III primary transcripts are removed by processing enzymes and (presumably) rapidly degraded, so that there may be no opportunity for capping, or capping that has occurred may not be detected. However, some Pol III transcripts retain their 5′ triphosphate and yet appear not to be capped. For example, yeast 5S rRNA is reported to have an unmodified triphosphate 5′ end (Hindley & Page, 1972). We suggest that 5S rRNA molecules are protected from capping by binding of the ribosomal protein L1 (previously called YL3) to a stem comprising the 5′ and 3′ termini of 5S rRNA (Nazar, 1979), and by assembly into ribosomes and export to the cytoplasm. Interestingly, approximately 10% of yeast 5S rRNA is variant in sequence (Piper et al., 1984) and binds L1 less well (Brow, 1987). Conceivably, this form of 5S rRNA is more susceptible to methylguanosine capping. Indeed, a minor fraction of yeast 5S rRNA has a phosphatase-insensitive 5′ terminus that can be capped in vitro after periodate oxidation and β-elimination (Wise et al., 1983), consistent with existence of a guanosine cap. A similarly minor fraction of yeast 5S rRNA is precipitated by anti-TMG antibody (Riedel et al., 1986). Our results suggest that the apparent existence of a small amount of TMG-capped 5S rRNA may not be an artifact of the assay, as was originally assumed. In fact, we cannot exclude the possibility that a small amount of wild-type U6 RNA is TMG capped (see Figs. 2 and 3). It will be of interest to determine to what extent methylguanosine capping of natural Pol III transcripts occurs in yeast cells.

MATERIALS AND METHODS

Plasmids and yeast strains

Construction of plasmids (pSE358; *TRP1 CEN4 ARS1*) and strains bearing the TATA-sub, TATAbox-sub, A1-sub, A2-sub, pseudo-wild-type, and 5′ΔNH6 alleles of *SNR6* have been described previously (Brow & Guthrie, 1990; Eschenlauer et al., 1993). The no-A mutant allele was made and introduced into yeast in the same manner, using the following mutagenic oligonucleotide: 5′-GTCCACGAACCGAAGCCC GCGAACAC-3′. The 5′Δ11 allele was generated by site-directed mutagenesis of pRS314-539H6 (*TRP1 CEN6 ARSH4*; Kaiser & Brow, 1995) with the following mutagenic oligonucleotide: 5′-GTCCACGAAGGGTTACATAGTTGCGAAAA AAAC-3′. SNR6 alleles on either pSE358 or pRS314 plasmids were transformed into yeast strains DAB017 or MWK023 (Eschenlauer et al., 1993) and transformants were plated on medium containing 5-fluoroorotate to select against the plasmid bearing the wild-type *SNR6* allele. For high copy expression, the 5′Δ11 allele was subcloned into pRS426 (*URA3 2μ* ORI; Christianson et al., 1992) by first isolating the *Eco*RI-*Sph*I fragment of pRS314-5′Δ11 and ligating it into *Eco*RI-*Sph*I-cut pRS317 (*LYS2 CEN6 ARSH4)*, then isolating the *Eco*RI-*Not*I fragment from pRS317-5′Δ11 and ligating it

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into *Eco*RI-*Not*I-cut pRS426. Strain MW670, which has a temperature-sensitive mutation in the gene encoding the large subunit of Pol III (Dieci et al., 1995), was kindly provided by Pierre Thuriaux (Centre d'Etudes de Saclay). MW670 has the genotype: *MAT***a** *ade2-101 lys2-801 ura3-52 trp1-Δ63 his3-Δ200 leu2-Δ1 rpc160-Δ1::HIS3* pC160-112 (*TRP1 CEN4 rpc160-112*). Strain YSB612, which contains a deletion of *CTL1* (Rodriguez et al., 1999), was kindly provided by Stephen Buratowski (Harvard Medical School).

Immunoprecipitation

IgG purified by Sephadex G-150 gel filtration from R1131 anti-TMG serum (Lührmann et al., 1982) was kindly provided by R. Lührmann. The anti-m^7G serum was kindly provided by T. Munns (Munns et al., 1982) via E. Lund (University of Wisconsin, Madison). Immunoprecipitations were done as described by Terns et al. (1992). Each immunoprecipitation mixture contained 25 μL swollen protein A-Sepharose CL-4B beads (Pharmacia) that had previously been incubated with 40 μg of IgG (or 4 μL of serum, for anti-m^7G precipitations), 50 U RNasin (Promega) and 5 μg total cellular RNA (Eschenlauer et al., 1993) in a 200-μL volume. Primer extension analysis was performed as described (Eschenlauer et al., 1993) using oligonucleotides U6D (complementary to nt 92–112 of yeast U6 RNA) and, in the case of anti-TMG precipitations, U4C (complementary to nt 32–52 of yeast U4 RNA).

Antibody gel-shift

2–5 μg total cellular RNA (Eschenlauer et al., 1993) and 50 fmol of ^{32}P-labeled oligonucleotide U6D and/or U4B (complementary to nt 140–158 of yeast U4 RNA) in 4 μL hybridization buffer (Li & Brow, 1993) were incubated at 90 °C for 1 min to dissociate U4/U6 complex, then at 37 °C for 15 min to form hybrids. For the experiment shown in Figure 5, oligonucleotide U6H (complementary to nt 13–30 of yeast U6 RNA) was used instead of U6D. After cooling on ice, 1 μL of IPP150 buffer (Terns et al., 1992) containing the indicated amount of H20 anti-TMG monoclonal antibody (Bochnig et al., 1987) was added and the mixture incubated at 4 °C for 1 h. After addition of 1.2 μL loading buffer (35% glycerol, 0.25% Bromophenol Blue, 0.25% Xylene Cyanol), the samples were electrophoresed at 4 °C, 30 V/cm on a 9% acrylamide (80:1 acrylamide:bisacrylamide) gel in 50 mM TBE (Li & Brow, 1993).

RNA analysis from Pol III mutant strain

The Pol III mutant strain MW670 (Dieci et al., 1995) was transformed with pRS426-5′Δ11, grown to mid-log phase in synthetic media lacking uracil at 25 °C, split into two cultures, one of which was shifted to the restrictive temperature of 37 °C, and grown for an additional 19 h, diluting each culture twofold with pre-warmed medium at 6 and 12 h. Total cellular RNA was prepared from the cell cultures as described previously (Treco, 1989). Approximately 2 μg (based on OD_{260} readings) of RNA and 50 fmol of ^{32}P-labeled oligonucleotide U6D, U4B, and U5B (complementary to nt 159–181 of yeast U5 RNA) in hybridization buffer were incubated at 90 °C for 1 min and then at 37 °C for 15 min to form hybrids (Li & Brow, 1993). After addition of 1.2 μL loading buffer, the samples were electrophoresed at 4 W on a 15-cm-tall, 1.5-mm-thick, 6% acrylamide native gel (30:1 acrylamide:bisacrylamide) in 50 mM TBE for 3 h at 4 °C.

ACKNOWLEDGMENTS

We thank Julie Eschenlauer for expert technical assistance, Reinhard Lührmann, Ted Munns, and Elsebet Lund for antibodies, Pierre Thuriaux and Stephen Buratowski for yeast strains, Wayne Versaw for constructing the no-A allele, Chris Solomon for constructing the 5′Δ11 allele, Ted Rasmussen and members of the Brow and Dahlberg labs for helpful discussions, and Elsebet Lund and Mike Martin for critical reading of the manuscript. This work was supported by Grant GM44665 from the National Institutes of Health (NIH) to D.A.B. S.K. was supported by NIH Training Grant GM08349. V.G. was supported by NIH Training Grant GM07215.

Received June 23, 1999; returned for revision July 26, 1999; revised manuscript received August 31, 2000

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