A minimal promoter for TFIIIC-dependent in vitro transcription of snoRNA and tRNA genes by RNA polymerase III

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Running Title : Minimal promoter for TFIIIC-dependent Pol III transcription

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The Saccharomyces cerevisiae SNR52 gene is unique among the snoRNA coding genes in being transcribed by RNA polymerase III. The primary transcript of SNR52 is a 250 nucleotide precursor RNA from which a long leader sequence is cleaved to generate the mature snR52 RNA. We found that the box A and box B sequence elements in the leader region are both required for the in vivo accumulation of the snoRNA. As expected the box B, but not the box A, was absolutely required for stable TFIIIC binding in vitro. Surprisingly, however, the box B was found to be largely dispensable for in vitro transcription of SNR52, while the box A-mutated template effectively recruited TFIIIB, but nevertheless it was transcriptionally inactive. Even in the complete absence of box B and both upstream TATA-like and T-rich elements, the box A still directed efficient, TFIIIC-dependent transcription. Box B-independent transcription was also observed for two members of the tRNAAsn(GTT) gene family, but not for two tRNAPro(AGG) gene copies. Fully recombinant TFIIIC supported box B-independent transcription of both SNR52 and tRNAAsn genes, but only in the presence of TFIIIB reconstituted with a crude B" fraction. Non-TFIIIB component(s) in this fraction were also required for transcription of wild type SNR52. Transcription of the box B-less tRNAAsn genes was strongly influenced by their 5'-flanking regions, and it was stimulated by TBP and Brf1 proteins synergistically. The box A can thus be viewed as a core TFIIIC-interacting element that, assisted by upstream TFIIIB-DNA contacts, is sufficient to promote class III gene transcription.

RNA polymerase (Pol) III synthesizes tRNA, 5S rRNA and a variety of other types of small nuclear and cytoplasmic RNAs. In general, the transcription of class III genes is under the control of internal control regions (ICR) characterized by discontinuous structures, with essential boxes separated by non-essential nucleotides (1). In the case of tRNA and 5S rRNA genes, the ICRs are highly conserved and comprise the binding sites for the general transcription factor TFIIIC (box A and box B) and for the 5S-specific factor TFIIIA (box C). Once assembled, TFIIIC recruits TFIIIB upstream of the transcription start site (TSS); TFIIIB in turn recruits Pol III for transcription initiation. The strong conservation of the ICRs likely reflects their dual function as both nucleation sites for transcription complex assembly and key determinants of tRNA and 5S rRNA structure. An indication of the constraints imposed on ICR by their overlapping structural and functional roles comes from the variability of promoter organizations displayed by the minority of class III genes not coding for tRNA and 5S rRNA. In some of these genes, the TFIIIC-interacting control regions (box A and box B) have been maintained within the transcribed region, and adapted to the structure of the small RNA without losing the transcriptional function. For example, in the Saccharomyces cerevisiae SCR1 gene, coding for the 7SL RNA, the box A and box B are both intragenic and involved in transcription, yet their sequences display distinguishing features as compared with the consensus of the tDNA box A
and box B (2). In the S. cerevisiae SNR6 gene, coding for the U6 snRNA, a non-canonical, yet transcriptionally relevant box A has been maintained within the transcribed region, while the box B is located downstream of the transcription termination site (3,4). Other class III genes entirely rely for transcription on upstream promoter elements, an organization that completely removes the need for the reciprocal adaptation of transcription control elements and RNA structure. One group of such genes, exemplified by the metazoan U6 snRNA and the human 7SK RNA genes, exploits upstream promoter elements similar to those of Pol II-transcribed genes (5). Other genes, such as RPR1, coding for the RNA component of S. cerevisiae RNase P (6), and the 7SL RNA genes of Trypanosomatidae (7,8), have adopted a promoter strategy that places a tRNA gene-like promoter, containing box A and box B, upstream of the mature 5'-end of the RNA. Such a strategy has recently been found to be employed for box C/D snoRNA expression in Arabidopsis thaliana and rice, where some snoRNAs are synthesized as tRNA-snoRNA precursors originated by Pol III transcription of tRNA-snoRNA dicistronic genes (9). Intriguingly, recent studies of the genome-wide localization of the Pol III transcription machinery in S. cerevisiae have identified the snoRNA-encoding SNR52 gene as a new class III gene, and sequence analysis has revealed the presence, upstream of the snoRNA coding sequence, of box A and box B elements that could together act as an external, tRNA gene-like promoter (10-12).

TFIIIC can specifically associate to target genes characterized by very different promoter configurations. This is due to the remarkable adaptability of this DNA-binding protein, which is related to the complexity and modular organization of its molecular structure. In particular, TFIIIC can bind to, and promote transcription of, templates characterized by very different box A-box B distances, from 20 to several hundreds bp (13-15). This property, together with the results of limited proteolysis (16) and electron microscopy studies (17), suggests that TFIIIC consists of two DNA binding modules separated by a flexible linker that can accommodate variously spaced A and B boxes. The TFIIIC-box B interaction gives the major contribution to the stability of the TFIIIC-DNA complex, while TFIIIC-box A contacts promote TFIIIB assembly and precise transcription initiation (18). A completely different mode of DNA binding by TFIIIC is that involved in 5S tRNA gene transcription. In this case, TFIIIC is recruited to the target gene essentially through protein-protein interaction with 5S DNA-bound TFIIIA (19). Adding to the complexity of TFIIIC action, DNA-bound TFIIIC has recently been shown to be involved in Pol III facilitated recycling in the yeast system (15,20). In some cases, discrepancies have been observed between box A/box B requirements for class III gene transcription in vitro and in vivo. Such discrepancies could be attributed, at least in part, to the fact that sub-optimally bound TFIIIC can efficiently recruit TFIIIB in vitro, but is impaired in its ability to counteract repressive chromatin assembly in vivo (2,21). In vitro, a defective interaction between TFIIIC and its split binding site can be tolerated and may result in only a moderate transcriptional impairment, provided that such interaction is stable enough to allow the formation of long-lived (kinetically trapped) TFIIIB-DNA complexes (22,23). This mainly occurs for mutations of the box A, that do not reduce dramatically the affinity of TFIIIC for the promoter (1). Though highly variable in sequence, the 5'-flanking region of box A and box B box-containing genes constitutes the binding site for TFIIIB, and may thus exert a strong influence on transcription efficiency (see (24) and references therein). In some eukaryotic genomes, the upstream regions of ICR-containing class III genes are characterized by the presence of a TATA box, starting at around ~30, that strongly contributes to transcription efficiency as an essential component of the promoter (25,26). In some cases, the upstream TATA element can even allow for TFIIIC-independent in vitro transcription (27,28). Whatever the mode of TFIIIC assembly, this factor acts in concert with TFIIIB to promote transcription of both TATA-less and TATA-containing class III genes. On TATA-less tRNA genes, TFIIIC binding initiates a series of protein-protein interactions ultimately resulting in TFIIIB assembly onto upstream DNA, with a key interaction taking place between the Tfc4 subunit of TFIIIC and the Brf1 component of TFIIIB (29,30).

In this study, we have carried out an extensive in vitro an in vivo analysis of SNR52 promoter architecture. Unexpectedly, we found that box B is dispensable while box A is essential for in vitro transcription of SNR52. This finding prompted us to re-evaluate the contribution of TFIIIC-box B interaction to promoter strength and its actual requirement for tRNA gene transcription.
MATERIALS AND METHODS

Amplification and cloning of DNA templates

The *S. cerevisiae* SNR52 gene was PCR-amplified from yeast genomic DNA (strain S288C) using the high fidelity Pfu DNA polymerase (Promega) and gene-specific pairs of oligonucleotide primers: SNR52_fw 5’- CTTTGAAAAAGATAATGTATGATTAGTC and SNR52_rev 5’- GCGTTCATACTGTCAAGGGTT for the amplified fragment (415 bp) contained the SNR52 transcribed sequence (260 bp) plus 110 bp of 5’-flanking and 45 bp of 3’-flanking sequences. The SNR52 Adown, Bdown, Tdown mutant variants were obtained by recombinant PCR (31). Two overlapping PCR primary products were generated using the SNR52_fw oligonucleotide in combination with 5’- GCGCACCTTTAGGGCTAGCCCAAGAAG and the SNR52_rev oligonucleotide in combination with the oligonucleotide 5’- CCTCCTGGGCTAGCCCTAAAGGTGC (mutagenic bases underlined). After gel purification, primary amplification products were mixed and used as templates in a subsequent amplification reaction, employing SNR52_fw and SNR52_rev as "outside" primers, that yielded the desired full-length secondary product. The same strategy was employed for the Bdown and the Tdown variants, using the following mutagenic oligonucleotides: 5’- GGAGAAGTTTCCAACGCCGAAACATGC and 5’- GCATGTTTCGGCGTTGGAACCTTCTCC for the Bdown mutation; 5’- GTAGGGTGTGAACGAATGCGCACC and 5’- GGTGCGCATTCGTTTCACACCCTAC for the Tdown mutation. The resulting DNA fragments were cloned into the SmaI site of the pUC-derived plasmid pNEB193 (New England Biolabs). For yeast transformation, all the inserts were subcloned into the shuttle vector pFL45S (32). 5’-mutated forms of SNR52 were obtained through amplification using the following mutagenic oligonucleotides as forward primers:

SNR52 5’-∆-58
SNR52 5’-∆-58mut
SNR52 5’-∆-7
SNR52_rev as reverse primer, and the proper plasmid template. 3’-truncated forms of SNR52 constructs (3’∆+60) were obtained through PCR amplification using the oligonucleotide 5’- GAAAAAATATGTAGGGTGAG as a reverse primer in combination with the different forward primers. The resulting DNA fragments were cloned into pNEB193. The plasmids containing the *S. cerevisiae* tRNA genes tN(GTT)CR, tN(GTT)NR, tP(AGG)CR and tP(AGG)NR have been described (24). Their 3’∆+64 truncated versions were prepared using the following reverse primers:

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5’- GAAAAAAAACAGATCTTGGGATTAC
for tDNAAsn(GTT) and
5’- GAAAAAAACCGGAGCTCCTCC
for tDNAPro(AGG).
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All the constructs were sequence-verified by dideoxy chain termination sequencing.

DNA binding assays

For DNase I footprinting analysis, a 415-bp SNR52 fragment, 5’ end-labeled with [γ-32P]ATP on the sense strand, was generated by PCR using 5′-labeled SNR52-fw and unlabeled SNR52_rev oligonucleotides as primers, and the wt, Adown, Bdown or Tdown versions of SNR52 as templates. 4 fmoles (~20000 cpm) of the purified fragment were incubated with 0.6 µg of TFIIIC, partially purified up to the DEAE Sephadex A-25 step following a previously described procedure (34), in 18 µl binding reaction mixtures containing 160 mM KCl, 10 µg/ml supercoiled pBlueScript-KS plasmid DNA, 0.1 mg/ml BSA, 5% glycerol, 20 mM HEPES pH 8, 4 mM MgCl2, 0.5 mM DTT. The complex was treated with 0.5 ng (0.05 U) of pancreatic DNase I (Amersham Biosciences, E2215Y type) for 1 minute at 25 °C. The digestion was stopped with 20 mM EDTA. Footprinting mixtures were phenol-extracted, ethanol-precipitated and fractionated on a 5% polyacrilamide, 7M urea sequencing gel, sided by products obtained from a dideoxy chain termination sequencing reaction conducted on wt SNR52 using the 5’-labeled SNR52-fw oligonucleotide. The gel was phosphorimagined with a Personal Imager FX (Bio-Rad). For gel retardation assays, 445-bp DNA fragments, containing the wt or mutated SNR52 transcription unit, were prepared by Bam HI-SphI digestion of the corresponding pNEB193 plasmids. The fragments were end-labeled with [α-32P]dATP and the Klenow fragment of DNA polymerase I (Promega), gel-purified and quantified by ethidium bromide staining. 3 fmoles of DNA fragment (15000 cpm) were then incubated 15 minutes at 25 °C with 0.6 µg of partially purified TFIIIC in a final volume of 15 µl. The standard binding reaction mixture contained 90 mM (NH4)2SO4, 13 µg/ml supercoiled pBlueScript-KS plasmid DNA, 1.3 mg/ml BSA, 10% glycerol, 10 mM Tris/HCl pH 8, 0.1 mM EDTA. In TFIIIC-containing reactions, DNA was first incubated...
with TFIIIC, then recombinant TFIIIB components (prepared as described below) were added in amounts ranging from 20 to 160 ng (see legend to Figure 4). The complexes were loaded on a pre-equilibrated native polyacrylamide gel (4% acrylamide, 0.048% bisacrylamide) containing 20 mM Tris/HCl pH 8, 1 mM EDTA, 5% glycerol. The running buffer contained 20 mM Tris/HCl pH 8, 1 mM EDTA, 5 mM β-mercaptoethanol. After loading, the gel was run 4-6 hours at 4°C, with frequent changes of running buffer. The gels were then dried and phosphorimaged.

In vitro transcription assays
Transcription of class III genes was reconstituted in vitro essentially as described (15,33). All reactions contained 0.6 µg of TFIIIC partially purified up to the DEAE Sephadex A-25 step (34), except that yeast nuclear extract (35) was used as a starting material; 40 ng of recombinant TBP and 80 ng of recombinant Brf1, both purified from overexpressing E. coli cells (34); and 10 ng of highly purified RNA polymerase III (15). As a source of Bdp1 protein, required to reconstitute TFIIIB activity, we used either 1.5 µg of B’ fraction, partially purified from chromatin pellets generated during yeast nuclear extract preparation (36), or 30 ng of recombinant 8His-Bdp1 protein purified from baculovirus-infected cells (15,37). Fully recombinant TFIIIC was expressed and purified as described (38). For the preparation of recombinant Nhp6A protein used in the experiment in Figure 7C, the S. cerevisiae NHP6A ORF was cloned into a modified version of pET28b containing an engineered Pme I restriction site into the polylinker to facilitate cloning of PCR products (39). The construct was transformed into BL21 Rosetta(DE3) E. coli cells (Novagen). Nhp6A expression was induced by adding IPTG (1 mM) and incubating for 2 hrs at 37°C. The 6His-Nhp6A protein in the soluble fraction was purified by chromatography on Ni-NTA resin (Qiagen) under non-denaturing conditions, following the manufacturer’s instructions. The template of in vitro transcription reactions (100-200 ng of class III gene-containing plasmid) was preincubated at 20°C for 20’ in a 45 µl reaction mixture (25 mM Tris/HCl, pH 7.9, 100 mM KCl, 5 mM MgCl₂, 8% (v/v) glycerol, 8 units of SUPERase-In RNase inhibitor (Ambion)) in the presence of transcription proteins (except Pol III). Pol III was then added together with cold NTPs (500 µM ATP, CTP, and GTP; 25 µM UTP) and 10 µCi of [α-32P]UTP (800 Ci/mmol, Amersham Biosciences), and multiple rounds of transcription were allowed to take place for 20’ at 20°C. Radiolabeled transcripts were separated on 6% polyacrylamide/7M urea gels, and visualized and quantified by phosphorimaging using a Personal Imager FX (BioRad). Primer extension analysis of in vitro produced transcripts was performed essentially as described (28). In vitro transcription reactions were carried out under the above described conditions, except that radiolabeled UTP was not included. Reaction products were then purified and used for primer extension analysis with Superscript III RNase H⁻ (Invitrogen) as a reverse transcriptase (200 U/reaction) and the 5’-end-ribonucleotide primer: 5’-GTATCAGAGATGGTTTCAGTCTAATG.

In vivo RNA analyses
The SNR52 null strain used for in vivo analyses is yM4585 (MATa his3A200 lys2-801 leu2-3,2-112 trp1-901 tyr1-501 URA3 ADE2 CANΔΔSNR52-HIS3) (40), in which the 92 bp sequence corresponding to the mature snr52 RNA, plus ~10 bp of upstream and downstream flanking sequences, have been deleted and substituted with the HIS3 marker gene. RNA extraction was performed as described (41) from cells grown to an OD₆₀₀ of 0.8-1 in the appropriate selective medium. 15 µg of total RNA were fractionated on a 6% polyacrylamide gel. 7M urea minigel, then transferred to a positively charged nylon membrane (Gene Screen Plus, Perkin Elmer). The filter was hybridised 15 hours at 42°C with a 5’-end-ribonucleotide oligonucleotide probe (5’-CAGAAGGAAGGCAACATAAGT) in 5x SSC, 5x Denhardt’s solution, 0.1 mg/ml denatured salmon sperm DNA, 0.5% (w/v) SDS, followed by two 10-minute washings at 30°C with 0.1% SDS and SSC 2X and then SSC 1X. The U3-specific oligonucleotide probe used for normalization was 5’-CAGACGGCATTTTTGTTTATAGTTTGTGATG-3’. Analyses is
null strain used for

RESULTS
Mutational analysis of the SNR52 promoter
The S. cerevisiae SNR52 gene has recently been identified as a novel class III transcription unit in which a snoRNA coding region is preceded by a leader region containing putative box A and box B control regions, as well as a TATA element located ~60 bp upstream of the box A (10). The sequence and organization of the SNR52 transcription unit are reported in Figure 1A. Both the box A and box B upstream of the snoRNA coding sequence can be easily recognized by analysis with the Pol3scan program (42), and have
been recently found to be conserved in hemiascomycetes (43). The distance between the two putative control elements (86 bp) is considerably higher than the average distance found in the yeast tRNA gene complement (37 bp). A curious feature of the transcription unit is the presence of a run of 6 consecutive T residues located a few bp downstream of the box A. Even though the T_{6} sequence is a strong termination signal for Pol III, we have recently shown that it behaves as an unusually weak terminator in the SNR52 context (33). The T_{6} element might be relevant for SNR52 expression, because it is also present in this position in the genomes of at least two other Saccharomyces species (S. paradoxus and S. mikatae). To start to characterize the SNR52 promoter, we amplified the SNR52 transcription unit from yeast genomic DNA, and we introduced mutations into the different putative control elements: box A, box B and the T run between them. The wt and mutant templates were assayed for their ability to support Pol III transcription both in vitro and in vivo. For in vitro assays, we used a Pol III-specific in vitro transcription system containing balanced amounts of recombinant TBP and Brf1 proteins, partially purified B^{+} and TFIIIC fractions and highly purified RNA polymerase III (34). For in vivo analysis, mutagenized SNR52 derivatives were inserted into the multicopy pFL455S vector (32) and transformed into a snr52::HIS3 null mutant strain (kindly provided by Sean Eddy (40)). The results of in vitro transcription analysis are shown in Figure 1B. Transcription of the wt template produced a 250 nt-long primary transcript and two smaller RNAs of 44-45 nt (lane 1). The shorter RNAs correspond to transcripts terminated at the internal T_{6} sequence; indeed, transcription of a mutant template in which the T run was interrupted produced higher levels of the full length transcript, while the short RNAs disappeared (lane 4). Transcript quantification, conducted by taking into account the different number of radiolabeled U residues incorporated into RNAs of different sizes, revealed only a slight decrease in transcription of the T-run mutant with respect to wt. In contrast, a double point mutation in the box A (CC in place of GG at positions +29, +30) abolished specific in vitro transcription (lane 2). Surprisingly, a G to C replacement in the box B, predicted to abolish TFIIIC binding and transcription (see for example (2)) only produced a 2-fold reduction of in vitro transcription with respect to wt (cf. lanes 1 and 3). Very similar in vitro results were obtained using a crude yeast nuclear extract as a source of transcription machinery (data not shown). The in vivo effects of the same mutations were analyzed by Northern blot after introducing the SNR52 variants into the snr52 null mutant strain. The results are shown in Figure 1C. A probe hybridizing with the mature snr52 snoRNA detected both the 90 nt mature product and the 250 nt precursor in the wt strain (lane 1). Box A and box B mutations both reduced the mature snR52 RNA to barely detectable levels (lanes 2 and 3; the precursor RNA was undetectable even after longer exposure), while the T run mutation did not significantly affect the levels of SNR52 transcription products (lane 4). The A box and B box are thus both required for SNR52 transcript accumulation in vivo, while in vitro the box B appears to be dispensable for transcription. Figure 1D shows the results of primer extension analysis conducted on in vitro produced SNR52 transcripts. Transcription initiated at a single position 18 bp upstream of the box A. As to the identity of this position, the gel shown in Figure 1D leaves some ambiguity between the T and the A at the center of a CTAG stretch. Since the general rule for Pol III is that it initiates transcription at a purine preceded by a pyrimidine residue (44,45), we assume that the A within the CTAG stretch is the actual TSS for SNR52. Figure 1D further shows that the TSS is not altered by mutation of the B box (lane 3) nor by interruption of the intragenic T run (lane 4). In spite of the very low levels of precursor transcript in vivo, we were also able to verify that the TSS observed in vitro is the same as in vivo (data not shown). 

**Binding of TFIIIC to the SNR52 control region**

The observation that box B mutational inactivation does not alter drastically the in vitro transcription capacity of SNR52 prompted us to directly analyze the TFIIIC-binding properties of wt and mutant SNR52 templates. Figure 2A shows the results of a DNase I footprinting analysis. A clear protection over the box B, but not over the box A, was observed with the wt template (cf. lanes 1 and 2). The same pattern was observed with the T stretch mutant (lanes 7 and 8). The lack of box A protection might be a consequence of the unusually large distance between box A and box B. This DNase I protection pattern was abolished upon mutational inactivation of the B box (lanes 5 and 6), while mutation of the box A produced a less dramatic decrease of box B protection (lanes 3 and 4). The results of gel retardation experiments, shown in Figure 2B, were in reasonable agreement with the footprinting analysis. As expected, mutation of the B box abolished TFIIIC binding to SNR52 (cf. lanes 1, 2 with lanes 5, 6), while T run disruption had no effect on TFIIIC binding under
the same conditions (lanes 7, 8). The box A mutation, causing a weakened box B protection in the footprinting assay, did not affect at all the amount of gel-retarded TFIIIC-DNA complex (lanes 3, 4). Since TFIIIC and DNA concentrations were very similar in footprinting and gel-retardation assays, the partial discrepancy between the results of the two assays might be due to a higher sensitivity of the footprinting assay in revealing sub-optimal TFIIIC-DNA interactions. From the results in Figures 1 and 2, we conclude that SNR52 can be efficiently transcribed by Pol III in vitro even in the absence of stable TFIIIC binding, while box A mutation abolishes transcription without severely affecting TFIIIC binding. One possible explanation of such unexpected behavior could be that TFIIIC, though required for SNR52 expression in vivo, is dispensable for its transcription in vitro (in this case, the box A requirement for in vitro transcription would be explained by assuming the existence of essential interactions between the A box and unknown transcription components). The results reported in Figure 2C, however, rule out this possibility, by showing that TFIIIC is absolutely required for in vitro transcription of both wt SNR52 and SNR52 variants mutated in the box B or at the internal T run (Bdown, Tdown).

Therefore, in the case of the Bdown template, TFIIIC promotes transcription without stably interacting with the SNR52 promoter.

The apparent extension of rRNA gene control regions can be manipulated by the conditions used to measure transcription in vitro. In particular, the use of high concentrations of templates can overcome in vitro the requirement for part of the control region (46). The template concentration used for the transcription assay in Figure 1 was 1 nM. In the experiment in Figure 3, the wt and mutant SNR52 templates were transcribed in vitro at concentrations ranging from 0.1 nM to 2 nM, in the presence of constant amounts of transcription proteins. Lowering the template concentration produced a clear decrease of transcription output in all cases (Figure 3B). Such a decrease, however, was most evident in the case of the B box mutant, whose transcription dropped by ~6-fold in going from 2 nM to 0.1 nM (cf. lanes 11 and 15 in Figure 3A; see also Figure 3B). By comparison, transcription of wt SNR52 was only 2.5-fold reduced at 0.1 nM with respect to 2 nM template (cf. lane 1 with 5; see Figure 3B). Therefore, the affinity of the transcription machinery for SNR52 is significantly reduced by the B box mutation. However, in vitro transcription of this template was still well detectable at sub-nanomolar template concentrations (lanes 11-13).

The discrepancies between the results of gel retardation and in vitro transcription assays with the Adown and Bdown SNR52 mutants were still observed when the two assays were carried out under identical reaction conditions with respect to the concentration of salt and glycerol (data not shown). We thus asked, by gel retardation assays, whether TFIIIB is able to associate with, and/or stabilize the TFIIIC-DNA complex at the Bdown promoter, and whether the Adown template, that is transcriptionally inactive, is able to recruit TFIIIB. The results of this experiment are shown in Figure 4. The complex between TFIIIC and the Adown template (lane 8) could be specifically supershifted by fully recombinant TFIIIB (lane 10), exactly as the wt complex did (lanes 2 and 4), while the Bdown template did not form any retarded complex, even in the presence of TFIIIB (lanes 13-18). We argue from these results that in vitro transcription of the Bdown mutant involves the formation of a TFIIIC/TFIIIB-DNA complex that is able to productively recruit Pol III in spite of being much less stable than canonical TFIIIC- and TFIIIB-containing preinitiation complexes, and that transcriptional inactivation of the Adown mutant is not due to reduced TFIIIB recruitment.

The SNR52 box A acts as an autonomous internal promoter element for Pol III transcription

The SNR52 transcription unit might contain, downstream of box A, a pseudo-B box that can be used when the natural B box is mutated. To address this point, we constructed a mutant version of SNR52 (SNR52 3′Δ+60) in which the transcription unit is truncated 24 bp downstream of box A by the insertion of a T9 termination sequence (Figure 5A). Since all the region downstream of box A, including box B, is absent from this template, the observed transcription output will be due to autonomous promoter activity of box A. Figure 5B, lane 2, shows that the truncated template containing a wt box A could be efficiently transcribed in vitro. Its transcription produced, as expected, a 45 nt transcript, corresponding to termination at the internal T9 run (this transcript disappeared when the T run was interrupted; see lane 4), and a longer RNA (~65 nt) corresponding to termination at the artificially inserted T9 terminator (lanes 2 and 4). The reaction in lane 1 was programmed with full length, wt SNR52 template. Quantification showed that SNR52 3′Δ+60 was transcribed as efficiently as wt SNR52. Lane 3 in Figure 5B further shows that the box A was absolutely required for transcription of the truncated template. To exclude the possibility that a pseudo-B box, present in the vector sequence downstream of the truncated
transcription unit, might provide a TFIIC anchoring site in the absence of the natural box B (47), we tested the transcription capacity of both the truncated, SNR52-3'Δ+60 template and the full length Bdown template (see Figure 1) after linearization at a restriction site located, within the vector polylinker, just downstream of the Ts terminator (in the case of SNR52-3'Δ+60) or of the natural SNR52 terminator (in the case of wt and Bdown SNR52). As shown in Figure 4C, plasmid linearization produced a general decrease in transcription efficiency with all the tested templates (wt SNR52, SNR52 Bdown and SNR52-3'Δ+60; cf. lanes 1, 3 and 5 with lanes 2, 4 and 6, respectively). However, both the linearized, box B-defective templates were transcribed almost as efficiently as the linearized, wt SNR52 gene (cf. lane 2 with lanes 4 and 6). The results in Figure 5 thus show that the SNR52 box A can autonomously (i.e., independently from the B box) mediate the productive association of the transcription machinery and accurate initiation at the SNR52 TSS. Transcription competition experiments, in which wt or truncated box B-less SNR52 templates were transcribed in vitro in the presence of increasing concentrations of a competitor tDNAle, further showed that box B-less SNR52 is significantly affected, with respect to the wt gene, in its ability to sequester the transcription machinery (data not shown).

It is well known that the 5'-flanking region of class III genes can strongly contribute to overall promoter strength (see (24) and references therein). It thus seemed likely that some features of the SNR52 5'-flanking region, such as the TATA element around position -40 and the T-rich region starting at -53, might influence SNR52 transcription, and possibly play an essential role, perhaps in synergy with the box A, in transcription of the Bdown or box B-less SNR52 templates. To test this possibility, we constructed variants of wt, Bdown and 3'Δ+60 (box B-less) SNR52 templates in which the 5'-flanking region was partially or totally mutated. The 5'-flanking sequences of these constructs are reported in Figure 6A. Figure 6B (lanes 1-4) shows that transcription of wt SNR52 was not affected by disruption of the TATA and T-rich elements, while the complete replacement of the 5'-flanking sequence by vector sequence produced a ~2-fold reduction in transcription (see also Figure 6C). The Bdown SNR52 template behaved similarly: its transcription was neither decreased by deletion of all the region upstream of -58 (lane 6), nor by disruption of both the TATA-like element at -40 and the T-rich element at -53 (lane 7), while the complete substitution of the 5'-flanking region with vector-derived sequence (lane 8) resulted in a 2-fold transcription decrease. A similar behavior was observed for the 3'Δ+60 (box B-less) template (lanes 9-10). The SNR52 box A thus behaves as a fully autonomous core promoter element for Pol III transcription.

**Box B-independent in vitro transcription of tRNA genes**

Since the box B is generally considered an essential promoter element for tRNA gene transcription, we wondered whether the box B-independent transcription of SNR52 reflects some unusual features of this particular template, related for example to its box A. We thus constructed box B-less versions of four different tRNA genes: two members of the tDNAAsn(GTT) family, tN(GTT)CR and tN(GTT)NR, and two members of the tDNAAsp(AGG) family, tP(AGG)CR and tP(AGG)NR. The tDNAs in each pair have identical ICRs but different 5'-flanking regions characterized by different affinities for TFIIB (24). As shown in Figure 7A, the A box region of the tDNAAsn pair is more similar to the SNR52 A box than the A box of the tDNAAsp pair. Box B-less, truncated versions of these tDNAs were constructed by inserting a terminator sequence (Tsla) 25 bp downstream of the A box and deleting all the downstream portion of the tDNA. The in vitro transcription efficiencies of the box B-less, truncated tDNAs were then compared with those of the corresponding wt templates. As expected on the basis of a previous study (24), the wt tN(GTT)NR template was transcribed less efficiently than wt tN(GTT)CR, due to differences in the 5'-flanking region (Figure 7B, cf. lanes 1-5 with lanes 11-15). Quite surprisingly, the box B-less versions of tN(GTT)CR (lanes 6-10) and tN(GTT)NR (lanes 16-20) both produced detectable levels of truncated tRNAAsn transcript. Remarkably, however, as revealed by quantification (taking into account the lower number of incorporated, 32P-labeled U residues into the truncated transcripts), the truncated, box B-less tN(GTT)CR was as efficiently transcribed as wt tN(GTT)CR (cf. lanes 6-10 with lanes 1-5), while the box B-less tN(GTT)NR was transcribed 4-5 times less efficiently than the corresponding wt tDNA (cf. lanes 16-20 with lanes 11-15). Therefore, at variance with SNR52 (see Figure 6), the ability of tDNAAsn(GTT) box A to promote transcription independently from the box B is significantly influenced by the 5'-flanking region. The box B-independent transcription of tDNAAsn(GTT) was still observed after linearization of the templates just downstream of the artificially inserted terminator (data not...
shown), thus excluding that residual transcription is due to pseudo-B boxes possibly present in the vector sequence downstream of the A box (47). Since the tN(GTT)NR upstream region has been characterized as a weak TFIIIB binding site (24), we asked whether increasing the concentration of TFIIIB components would specifically enhance the transcription of box B-less tN(GTT)NR. In the experiment in Figure 7C, individual recombinant TFIIIB components, as well as all different combinations of them, were provided in a 5-fold excess with respect to standard transcription conditions. The transcription of box B-less versions of both tN(GTT)CR and tN(GTT)NR was not stimulated by an excess of any individual TFIIIB component (lanes 1-4 and 10-13), nor by supplementation of the high-mobility-group Nhp6 protein (lanes 5 and 14), that has been previously reported to stimulate SNR6 transcription by Pol III (48,49) and to influence transcription start site selection on yeast tRNA genes (50). When TBP and Brf1 proteins were supplemented together, however, ~4-fold and ~3-fold stimulations were observed for box B-less tN(GTT)CR and tN(GTT)NR, respectively (lanes 6 and 15). Stimulations were slightly less effective when excess Bdp1 protein was also added together with TBP and Brf1 (lanes 9 and 18). TFIIIB component supplementation did not level the transcriptional difference between the two templates. Under all the conditions tested, the box B-less tN(GTT)NR template was always more than 10 times less transcribed than box B-less tN(GTT)CR. A completely different behaviour, in box B-independent transcription, was observed in the case of tRNA^Phe^(AGG) genes. As shown in Figure 7D, tP(AGG)NR was a better template than tP(AGG)CR, as it was very efficiently transcribed at the lowest concentration tested (0.1 nM, lane 11). When the isocoding tP(AGG)CR was used at the same concentration, no transcription was observed (lane 1), most likely due to the lower transcriptional strength of its upstream region (24). In spite of the high affinity of tP(AGG)NR for the transcription machinery, however, its box B-less variant could not be transcribed (lanes 16-20). The box B-less version of tP(AGG)CR was also transcriptionally inactive (lanes 6-10). The ability to promote transcription complex assembly in vitro in a B box-independent fashion is thus not a general property of tDNA box A elements.

SNR52 and box B-independent transcription in the presence of all-recombinant transcription factors. A recent paper has reported the reconstitution of in vitro class III gene transcription from fully recombinant TFIIIB and TFIIIC (38). We thus asked whether SNR52 can be transcribed in a system reconstituted with all-recombinant factors, and whether fully recombinant TFIIIC and TFIIIB are sufficient to support box B-independence transcription. In the experiment in Figure 8A, wt, Bdown and box B-less versions of SNR52 were transcribed in vitro in the presence of either native or recombinant TFIIIC in combination with TFIIIB either in a fully recombinant form or containing the crude B” fraction in place of recombinant Bdp1 protein. When B”-containing TFIIIB was used, recombinant TFIIIC could replace native TFIIIC in the transcription of both wt and box B-mutated SNR52 templates (cf. lanes 3, 7 and 11 with lanes 4, 8 and 12, respectively). When fully recombinant TFIIIB was used, however, neither native nor recombinant TFIIIC could support transcription of any of the templates (lanes 1, 2, 5, 6, 9 and 10). The B” fraction thus appears to contain, in addition to Bdp1 protein, additional component(s) required for SNR52 transcription. Such components seem not to be required for TFIIIB assembly onto SNR52, because the SNR52-TFIIIC complex could be specifically supershifted by all-recombinant TFIIIB in gel-retardation assays (see Figure 4). We also tested the ability of different TFIIIC and TFIIIB combinations to transcribe wt and box B-less versions of tN(GTT)CR. Figure 8B shows that, as expected (38), the wt tN(GTT)CR template could be transcribed in the presence of all-recombinant TFIIIC and TFIIIB, albeit at very low efficiency (lane 1). tN(GTT)CR transcription was strongly stimulated when B” fraction replaced recombinant Bdp1 (cf. lanes 1 and 2). Recombinant TFIIIC could also support transcription of the box B-less tN(GTT)CR template (lane 5). With this template, the requirement for non-Bdp1 component(s) in the B” fraction was even more evident, as no transcription was observed in the presence of fully recombinant factors (lane 4).

**DISCUSSION**

In this study, we show that SNR52 transcription by RNA polymerase III depends on a box A/box B combination within the 5’ leader region that binds TFIIIC and allows for efficient transcription initiation at a site located ~160 bp upstream of the mature snoRNA coding sequence. Both A and B box are required for in vivo accumulation of the mature snoRNA. Since the ICR sequences do not appear in the mature RNA product, the reduced snoRNA levels observed in vivo with Adown and Bdown SNR52 templates are most likely due to
impairment of transcription, and not to reduced stability of the RNA products. Quite unexpectedly, in vitro analysis revealed that the SNR52 box B, though essential for TFIIIC binding, is dispensable for in vitro transcription, while the box A, giving only a minor contribution to TFIIIC binding affinity, is essential for transcription. The dispensable character of box B in vitro was also observed for two tRNA\textsuperscript{Asn}(GTT) genes, whose box A behaved as an autonomous intragenic promoter element able to nucleate TFIIIC-dependent TFIIIB assembly. In contrast, the B box of two tRNA\textsuperscript{Pro}(AGG) genes could not be removed without inactivating the templates. The B box-independent in vitro transcriptional activity of the tRNA\textsuperscript{Asn} genes, but not of SNR52, was found to be strongly influenced by the 5'-flanking region.

The finding that tRNA gene transcription can take place in the absence of box B is not unprecedented. Early studies, analyzing transcription of different tRNA gene fragments in crude extracts, reported that box B-less, truncated tRNA genes, at high concentrations, can initiate transcription in vitro at their normal site, albeit very inefficiently (46,51-53) (reviewed in (1)). Our data strengthen and extend these observations by showing that box B-independent transcription takes place at a remarkable efficiency in vitro even when the template concentration is in the sub-nanomolar range. The apparent equilibrium constant for yeast TFIIIC binding to the SUP4 tRNA\textsuperscript{Asn} gene has been estimated to be \( \sim 1.9 \times 10^9 \text{ M}^{-1} \), and to drop by 370 fold, to \( 5 \times 10^6 \text{ M}^{-1} \), upon box B mutational inactivation (54). The fact that a box B-less tDNA, like tN(GTT)CR 3'\( \Delta^+64 \), is transcribed at wild type levels even at a template concentration of \( 1 \times 10^{-10} \text{ M} \) indicates that, in our in vitro transcription conditions, a TFIIIC-box A interaction, though very weak, is nevertheless sufficient to nucleate TFIIICB assembly. At variance with SNR6, whose TATA box-dependent, box B-independent transcription could not be observed in crude extracts, due to the presence of inhibitory chromatin components normally counteracted by TFIIIC (55), box B-independent transcription of both SNR52 and tDNA\textsuperscript{Asn}(GTT) was also observed with yeast nuclear extract as a source of Pol III transcription machinery (data not shown). Our data thus suggest that, as previously pointed out (1), the stability of the TFIIIC-DNA complex, whose major contribution comes from interaction with the B box, is not absolutely required for transcription. On the other hand, what emerges from promoter analysis of SNR52 is the centrality of box A in directing productive TFIIICB assembly and transcription initiation. We found that the SNR52 box A is absolutely required for transcription not only in vivo but also in vitro. This observation contrasts with all the previously reported box A mutations, whose effects on Pol III transcription were never reported to be as severe as in the case of Adown SNR52 (1). Accordingly, early studies of the effects on tRNA gene transcription of base substitutions in the ICRs led to the common notion that tRNA gene promoter strength is principally determined by the B box, while the TFIIIC-box A interaction plays a secondary role in favoring TFIIICB assembly and start site selection (47,56-58). A more recent analysis of promoter organization of the non-tRNA class III gene SCRI confirmed the primary role of the box B in determining the promoter strength in vitro, but also pointed out the importance of TFIIIC-box A interaction in counteracting repressive chromatin assembly in vivo (2). A chromatin antirepression role of the TFIIIC-box A interaction has also been highlighted for the S. cerevisiae SNR6 gene (21). In the case of SNR52, in the absence of other transcription factors, the B box appears to be the main determinant of TFIIIC binding affinity (TFIIIC binding is abolished by a B box point mutation, while it is only slightly affected by A box disruption), yet the box A is essential for transcription, and it can even work independently from box B in directing efficient in vitro transcription. The results of gel-retardation analysis suggest that the SNR52 box A mutation does not impair TFIIIC binding nor TFIIICB recruitment. Its drastic effect on transcription thus likely results either from TFIIICB misplacement, perhaps directed by other, non-optimal box As, of from an unexpected involvement of box A in post-recruitment step of the transcription cycle.

In the case of box B-less tDNAs, the autonomous promoter activity of the box A was found to be sequence context-dependent. In particular, the analysis of two box B-less tDNA\textsuperscript{Asn}(GTT) templates differing for their upstream regions revealed that, in the absence of stable TFIIIC binding mediated by the B box, the box A and 5'-flanking region act synergistically to promote the assembly of a functional pre-initiation complex. Moreover, the sequence of the box A itself might be relevant for autonomous promoter activity, as suggested by the observation that the SNR52 and tDNA\textsuperscript{Asn}(GTT) A boxes, both supporting box B-independent transcription, are more similar in sequence to each other than to the tDNA\textsuperscript{Pro}(AGG) box A, that is unable to support box B-independent transcription (see Figure 7). A synergistic action of box A and upstream sequence elements in transcription initiation has previously been observed. For example, it has been shown
that SNR6 transcription initiation in vivo is insensitive to mutation of the upstream TATA box, unless the box A is simultaneously mutated (59). It is also known that TSS selection on tRNA genes is co-directed by a TFIIIB component, TBP, and by the most upstream, A box-interacting portion of TFIIIC (60). By contrast, the position of the B box downstream of box A does not affect TSS selection, and transcription efficiency is generally unaffected even at very large A box to B box distances (14,15,61). By underlining the partially dispensable character of box B for transcription in vitro, our data contribute to define the upstream moiety of tRNA genes, comprising the box A and the 5'-flanking sequence, as a minimal core promoter module where all the key interactions leading to transcription initiation can take place. With this respect, it was interesting to find that box B-independent tDNA transcription could be strongly stimulated by simultaneously increasing the concentrations of both TBP and Brf1, but not of either component alone. Such a cooperative effect is in agreement with the fact that these two components interact with each other (62), with TFIIIC subunits (29,63) and with upstream DNA (64). In particular, by binding to both TBP and the τ131 subunit of TFIIIC, Brf1 could contribute to generate an extension of the DNA binding surface of the box A-interacting TFIIIC domain, thus conferring to the transcription factor-DNA complex sufficient stability in order to perform Pol III recruitment. Within this scenario, the role of the B box in the canonical TFIIIC-DNA interaction would simply be to contribute additional stability to the transcription complex that assembles on the core promoter elements. It is interesting to note that such a minimal TFIIIC/TFIIIB assembly module is strongly reminiscent of the composite organization of the RNA polymerase II core promoter. A Pol II core promoter usually comprises some combination of an upstream moiety, containing a TATA box preceded by a BRE (TFIIB-recognition element), an Initiator element surrounding the TSS, and a downstream promoter element (DPE), conserved from Drosophila to humans, that is located at +30 with respect to the TSS (65). Correspondingly, in tRNA genes, an Initiator-like element surrounding the TSS (and characterized, in some genomes, by the presence of a CAA triplet (24)) is flanked immediately upstream by a TFIIIB-binding region often carrying a TATA-like element and, downstream, by an A box element whose conserved positions are concentrated in a region roughly extending from +20 to +30 with respect to the TSS. These similarities in core promoter organization of Pol II and Pol III transcription units, together with our finding that 5’ flank and A box are sufficient to promote TFIIIC-dependent Pol III transcription, suggest a functional parallelism between TFIIID, that contacts DNA both upstream and downstream of the TSS through interactions with the TATA, Initiator and DPE elements, and the DNA recognition module composed of TFIIIB and the box A-interacting portion of TFIIIC.

Finally, we note that the availability of fully recombinant TFIIIB and TFIIIC factors allowed us to reveal the absolute requirement for additional activities, different from the well characterized TFIIIC and TFIIIB components, both for SNR52 transcription and for box B-independent tDNA transcription. This observation adds to a growing list of reports suggesting that the activity of the yeast Pol III transcription system can be directly modulated by different effectors (15,35,38,48,50,66). In particular, the possibility is suggested that SNR52 transcription requires (a) novel gene-specific factor(s), that might be also involved in transcription of the other snoRNA genes by RNA polymerase II, thus providing a way for coordinate expression of these genes by two different RNA polymerases.
ACKNOWLEDGMENTS

We thank Sean Eddy for the snr52 null mutant strain, Tiziana Lodi for plasmid vectors, Joël Acker for recombinant TFIIIC and André Sentenac for helpful suggestions. This work was supported by the Human Frontier Science Program Organization (grant RGY0011/2002-C to G.D.) and by the Italian Ministry of Education, University and Research (FIRB and COFIN Programs).

FOOTNOTES

1 Abbreviations used are: ORF, open reading frame; Pol, RNA polymerase; TF, transcription factor; ICR, internal control region; TSS, transcription start site; wt, wild type; nt, nucleotides; bp, base pairs; r, recombinant.

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FIGURE LEGENDS

Figure 1. Transcription properties of the SNR52 promoter. (A) Sequence of the SNR52 transcription unit. The sequence corresponding to the mature snoRNA is underlined. The upstream TATA element, box A, box B and T runs are boxed. The base substitutions introduced into the SNR52 leader region to generate the Adown, Bdown and Tdown mutants are reported below the sequence (with the original bases in bold character). The TSS (+1), determined experimentally in this study (see Fig. 1D), is in bold italic character and underlined. (B) In vitro transcription of wt and mutant SNR52 templates. The migration positions of the 250 nt, full length transcript (250) and of the short transcripts terminated at the internal T run (45) are indicated on the left. The transcriptional output of each reaction, relative to the output (arbitrarily set to 100) of the wt template, is reported below each lane (Tnx). (C) Northern analysis of in vivo transcription products of wt and mutant SNR52 templates. Total RNA extracted from the snr52 null mutant strain transformed with the indicated templates (carried by the pFL45S vector) was gel fractionated and probed with a radiolabeled oligonucleotide complementary to the mature snoRNA. The migration positions of the 250 nt, precursor RNA (250) and of the mature snoRNA (90) are indicated on the right. The same blot was hybridized with a probe specific for the U3 snRNA, used as an internal standard. The normalized RNA levels of mature snR52 snoRNA measured in each case are reported below the gel image (RN4); the values are expressed as percentages of the RNA levels obtained with wt SNR52. (D) The RNA products of in vitro transcription reactions programmed with either wt or mutant SNR52 templates were subjected to primer extension analysis (lanes 1-4). Shown in lanes 5-8 are the results of sequencing reactions conducted using the same 5'-labeled oligonucleotide utilized for primer extension. The sequence of the non-transcribed DNA strand around the start site (+1) is indicated on the right.

Figure 2. DNA binding properties of the SNR52 promoter region. (A) DNA fragments containing the wt (lanes 1, 2), Adown (lanes 3, 4), Bdown (lanes 5, 6) or Tdown (lanes 7, 8) SNR52 promoters, radiolabeled on the sense strand, were incubated either in the presence (lanes 2, 4, 6 and 8) or in the absence (lanes 1, 3, 5 and 7) of TFIIIC, digested with DNase I and processed as described in Materials and methods. Shown in lanes 9-12 are the results of dideoxy chain-termination sequencing reactions conducted with the same 5'-labeled oligonucleotide utilized to amplify the SNR52 fragments. The position of the box A, box B and internal T run are reported on the right. (B) Radiolabeled DNA fragments containing the wt (lanes 1, 2), Adown (lanes 3, 4), Bdown (lanes 5, 6) or Tdown (lanes 7, 8) SNR52 promoters were incubated either in the presence (lanes 2, 4, 6 and 8) or in the absence (lanes 1, 3, 5 and 7) of TFIIIC, followed by electrophoretic fractionation of TFIIIC-bound and free DNA molecules on a native polyacrylamide gel. The percentages of bound DNA (with respect to total DNA) in each TFIIIC-containing reaction are reported below the gel image. (C) The indicated SNR52 template variants were transcribed in vitro either in the presence (lanes 2, 4, 6 and 8) or in the absence (lanes 1, 3, 5 and 7) of TFIIIC. The migration positions of the 250 nt, primary transcript (250) and of the short transcripts terminated at the internal T run (45) are indicated on the right (the two panels derive from the same gel image).

Figure 3. In vitro titration of wt and mutant SNR52 templates. (A) The wt (lanes 1-5), Adown (lanes 6-10) or Bdown (lanes 11-15) SNR52 templates were used, at increasing concentrations (indicated above the lanes) to program in vitro transcription reactions. The migration position of the 250 nt primary transcript (250) and of the short transcripts terminated at the internal T run (45) are indicated on the left. (B) The diagram derives from phosphorimaging quantification of the gel in panel A. Filled and open circles refer, respectively, to the wt and Bdown templates.

Figure 4. Gel-retardation analysis of TFIIIB recruitment by SNR52 promoter mutants. Radiolabeled DNA fragments containing the wt (lanes 1-6), Adown (lanes 7-12), Bdown (lanes 13-18) SNR52 promoters were
incubated with: partially purified TFIIIC alone (lanes 2, 8 and 14); TFIIIB alone, reconstituted with 20 ng rBdp1, 80 ng rBrf1, 40 ng rTBP (1X rTFIIIB; lanes 5, 11, 17); TFIIIB alone, reconstituted with 2-fold higher amounts of the three components (2X rTFIIIB; lanes 6, 12, 18); TFIIIC plus 1X rTFIIIB (lanes 3, 9, 15); TFIIIC plus 2X rTFIIIB (lanes 4, 10, 16). No factor was added in lanes 1, 7, 13. Protein-bound and free DNA molecules were separated by electrophoretic fractionation of on a native polyacrylamide gel. The migration positions of free DNA, TFIIIC-DNA and TFIIIB-TFIIIC-DNA complexes are indicated on the left.

**Figure 5.** *In vitro* transcription of box B-less SNR52 templates. (A) Sequence of the box B-less SNR52 template (SNR52_3′Δ+60). The transcribed region is in bold character. The box A and the artificially introduced T₉ terminator are boxed. The TSS (+1) is in italic character and underlined. The base substitutions introduced to generate the Adown and Tdown versions of the truncated template are reported below the sequence. (B) *In vitro* transcription of box B-less SNR52 templates, either without base substitutions in the transcribed region (A’T’, lane 2) or with mutations in box A (Ad, lane 3) or T run (Td, lane 4). The full-length, wt SNR52 template was used to program the reaction in lane 1. The migration positions of the internally terminated (45) and full-length (250) SNR52 transcripts, as well as of transcripts terminated at the T₉ terminator introduced to generate the B box-less templates (65) are indicated on the left. (C) The wt (lanes 1, 2), Bdown (lanes 3, 4) or box B-less (lanes 5, 6) SNR52 templates, either in a circular supercoiled form (lanes 1, 3, 5) or in an EcoRI-linearized form (lanes 2, 4, 6), were used to program *in vitro* transcription reactions. The transcriptional output of each reaction, relative to the output (arbitrarily set to 100) of the wt, supercoiled template (lane 1), is reported below each lane (Txn).

**Figure 6.** Role of the 5′-flanking region in SNR52 transcription *in vitro*. (A) Sequence of the 5′-flanking regions of SNR52 upstream variants. In the 5′A-58 variant, all the natural SNR52 sequence upstream of position −58 (with respect to the TSS), was deleted. The upstream T-rich and TATA elements are in bold character. The base substitutions that have been introduced to generate the 5′Δ-58mut template variants are underlined. The TSS (+1) is in italic bold character. In the case of the 5′Δ-7 variants, the plasmid-derived sequence that replaces the original 5′-flanking sequence is in italic character. (B) SNR52-derived templates containing wt (lanes 1-4), Bdown (lanes 5-8) or 3′Δ+60 (lanes 9-10) transcribed regions in combination with wt (lanes 1, 5, 9), 5′Δ-58 (lanes 2, 6), 5′Δ-58mut (lanes 3, 7) or 5′Δ-7 (lanes 4, 8, 10) upstream regions, were used to program *in vitro* transcription reactions. Reactions in lanes 1-8 contained standard amounts of TFIIIB components. Reactions in lanes 9-10 contained reduced amounts (0.8 µg) of B” fraction, as higher amounts were found to be selectively inhibitory for the 3′Δ+60/5′Δ-7 template. The migration positions of the internally terminated (45) and full-length (250) SNR52 transcripts, as well as of transcripts terminated at the T₉ terminator introduced to generate the B box-less templates (65) are indicated on the left and on the right. (C) The bar plot derives from phosphorimaging quantification of the experiments shown in panel B and of duplicate experiments conducted in parallel. The different templates are grouped, on the x-axis, according to the sequence of their transcribed region (wt, Bdown or 3′Δ+60). The average transcription levels (expressed as percentage of the levels observed, within each group, with the template having a wt upstream region) are reported on the y-axis. The different bar colours refer to the different 5′-flanking regions (wt, 5′Δ-58, 5′Δ-58mut, 5′Δ-7) as indicated in the inset. Error bars indicate the standard error.

**Figure 7.** *In vitro* transcription of B box-less tRNA genes. (A) Sequence comparison of the box A elements present in the SNR52 gene and in the tN(GTT) and tP(AGG) gene families of *S. cerevisiae*. The base positions shaded in orange are conserved in all the three sequences; bases shaded in green are only conserved between SNR52 and tN(GTT) box A sequences, while those shaded in violet are only conserved between SNR52 and tP(AGG) box A sequences. (B) Increasing concentrations (from 0.1 nM to 2 nM) of either tN(GTT)CR, in its wt (lanes 1-5) or B box-less truncated (lanes 5-10) versions, or tN(GTT)NR, in its wt (lanes 11-15) or B box-less truncated (lanes 16-20) versions, were used to program *in vitro* transcription reactions. The migration positions of the full-length, pre-tRNA⁴⁵m transcript, and of the truncated RNA produced from box B-less templates, are indicated on the left. (C) The tN(GTT)CR 3′Δ+64 (lanes 1-9) and tN(GTT)NR 3′Δ+64 (lanes 10-18) templates were transcribed *in vitro* in the presence of standard amounts of partially purified TFIIIC and B”-containing reconstituted TFIIIB (stTFIIIB) supplemented with: 200 ng rTBP (lanes 2, 11); 400 ng rBrf1 (lanes 3, 12); 150 ng rBdp1 (lanes 4, 13); 100
ng rNhp6A (lanes 5, 14); and all the different combinations of TFIIIB components in the above reported amounts, as indicated above the lanes. (D) Increasing concentrations (from 0.1 nM to 2 nM) of either tP(AGG)CR, in its wt (lanes 1-5) or B box-less (lanes 6-10) versions, or tP(AGG)NR, in its wt (lanes 11-15) or B box-less (lanes 16-20) versions, were used to program in vitro transcription reactions. The migration position of the full-length, pre-tRNA^{3′} transcript is indicated on the left.

**Figure 8. SNR52 and box B-independent transcription in the presence of all-recombinant transcription factors.** (A) The wt (lanes 1-4), Bdown (lanes 5-8) or 3’Δ+60 SNR52 templates were transcribed in vitro in the presence of either 100 ng of recombinant TFIIIC (rTFIIIC; lanes 1, 3, 5, 7, 9, 11) or 0.6 µg of partially purified native yeast TFIIIC (natC; lanes 2, 4, 6, 8, 10, 12) in combination with either fully recombinant TFIIIB (lanes 1, 2, 5, 6, 9, 10) or B’'-containing TFIIIB (lanes 3, 4, 7, 8, 11, 12). The migration positions of the internally terminated (45) and full-length (250) SNR52 transcripts, as well as of transcripts terminated at the T9 terminator introduced to generate the B box-less templates (65) are indicated on the left and on the right. (B) The tN(GTT)CR (lanes 1-3) and tN(GTT)CR 3’Δ+64 (lanes 4-6) templates were transcribed in vitro in the presence of either fully recombinant TFIIIC (rTFIIIC; lanes 1, 2, 4, 5) or partially purified native yeast TFIIIC (natC; lanes 3, 6) together with either fully recombinant TFIIIB (lanes 1 and 4) or B’'-containing TFIIIB (lanes 2, 3, 5, 6). The migration positions of full-length or truncated tDNA^Asn transcripts are indicated on the left and on the right, respectively.
Figure 1

A

CTTTGAAAAGATAATGTATGATTATGCTTTCACTCATATTATACAGAAACTTGATGTTTTC
TATA box
T run
box A
+1
A

TCGGCTAGGCTAAAGGGCTGCCGGTTTTCGAGTATATACAAGGTGATTACATGTACGTTTGAAGTACAACTCT
box B

AATCGAAAGATTTTAGGATTAGAAAAACTTATGTTGCCCTCTCTGAAAAATTATTCTTTTT

B

SNR52 Adown Bdown Tdown
250

45

1 2 3 4

1 2 3 4

Txn: 100 0 48 69

C

RNA: 100 0.7 1.1 90

D

SNR52 Adown Bdown Tdown

G T A C

C T T C T

A (+1) G
Figure 2
Figure 4

|        | SNR52 | Adown | Bdown |
|--------|-------|-------|-------|
| TFIIIC | -     | +     | +     |
| rTFIIIB| -     | -     | +     |
| C-B-DNA| -     | -     | -     |
| C-DNA  | -     | -     | -     |
| free DNA| -     | -     | -     |

Table showing the presence (+) or absence (-) of TFIIIC, rTFIIIB, C-B-DNA, C-DNA, and free DNA in SNR52, Adown, and Bdown conditions.
A

SNR52_3'Δ+60

CTTTGAAAAGATAATGTATGATTATGCTTTACTCATATTTATACAGAAAACCTTGATGTTTTC
TTTCGAGTATATACAAAGTGATTACATGTACGTATTGAAGTACAACTCTCATCT

box A

CCTCGTTGGGTAGGTTAAGGGTGGCATTTCACACCCCTACATTTTTTTTTTTTTTT

B

SNR52

3'Δ+60

A'T' Ad Td

C

SNR52 Bdown 3'Δ+60

Eco RI

1 2 3 4

1 2 3 4 5 6

Txn: 100 47 77 39 95 31
Figure 6

A

5'Δ-58
TGATGTTTTCTTGAGGTATATATACAAGGTGATTACATGTACGTGGTTGGAAGTTACAAACTCTAGA

5'Δ-58mut
TGATGTTTCCTCTTCAGGTATTACATGTACGTGGTTGGAAGTTACAAACTCTAGA

5'Δ-7
cccagtcacgcgtttgtaaaacgacggccagtgaattcgagctcgtaccCAACTCTAGA

B

|         | 5 wt | 5'Δ-58 | 5'Δ-59mut | 5'Δ-7 |
|---------|------|--------|-----------|-------|
| WT      |      |        |           |       |
| Bdown   |      |        |           |       |
| 250     |      |        |           |       |
| 45      |      |        |           |       |

|         | 5 wt | 5'Δ-58 | 5'Δ-59mut | 5'Δ-7 |
|---------|------|--------|-----------|-------|
| 3'Δ+60  |      |        |           |       |
| 65      |      |        |           |       |
| 45      |      |        |           |       |

C

Relative transcription

| Template          | WT | Bdown | 3'Δ+60 |
|-------------------|----|-------|--------|
| wt                |    |       |        |
| 5'Δ-58            |    |       |        |
| 5'Δ-58mut         |    |       |        |
| 5'Δ-7             |    |       |        |
Figure 7

A

**box A**

tN(GTT)\[tN(GTT)CR\] tN(GTT)NR 3’

\[\Delta +64\]

pre-tRNA\[^{\text{Asn}}]\n
truncated

B

| Template       | tN(GTT)CR | tN(GTT)CR 3’Δ+64 | tN(GTT)NR | tN(GTT)NR 3’Δ+64 |
|----------------|-----------|-----------------|-----------|-----------------|
| pre-tRNA\[^{\text{Asn}}]\ |           |                 |           |                 |
| truncated      |           |                 |           |                 |

C

| TBP | Brf1 | Bdp1 | Nhp6 | stTFIIIB |
|-----|------|------|------|----------|
| +   | +    | +    | +    | +        |
| +   | +    | +    | +    | +        |
| +   | +    | +    | +    | +        |
| +   | +    | +    | +    | +        |

D

| Template       | tP(AGG)CR | tP(AGG)CR 3’Δ+64 | tP(AGG)NR | tP(AGG)NR 3’Δ+64 |
|----------------|-----------|-----------------|-----------|-----------------|
| pre-tRNA\[^{\text{Pro}}]\ |           |                 |           |                 |
Figure 8

A

|                | SNR52 | Bdown | 3' Δ+60 |
|----------------|-------|-------|---------|
| rTBP, rBrf1, B'' | +     | +     | +       |
| natC           | +     | +     | +       |
| rTBP, rBrf1, rBdp1 | +     | +     | +       |
| rC             | +     | +     | +       |

B

|                | tN(GTT)CR | 3'Δ+64 |
|----------------|-----------|--------|
| rTBP, rBrf1, B'' | +         | +      |
| natC           | +         | +      |
| rTBP, rBrf1, rBdp1 | +      | +      |
| rC             | +         | +      |

pre-tRNA^{Asn}_truncated
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J. Biol. Chem. published online June 19, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M513814200

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