Eukaryotic RNA polymerase (Pol) III terminates transcription at short runs of T residues in the coding DNA strand. By genomic analysis, we found that T₅ and T₄ are the shortest Pol III termination signals in yeasts and mammals, respectively, and that, at variance with yeast, oligo(dT) terminators longer than T₅ are very rare in mammals. In *Saccharomyces cerevisiae*, the strength of T₅ as a terminator was found to be largely influenced by both the upstream and the downstream sequence context. In particular, the CT sequence, which is naturally present downstream of T₅ in the 3’ flank of some tDNAs, was found to act as a terminator-weakening element that facilitates translocation by reducing Pol III pausing at T₅. In contrast, tDNA transcription termination was highly efficient when T₅ was followed by an A or G residue. Surprisingly, however, when a termination-proficient T₅ signal was taken out from the tDNA context and placed downstream of a fragment of the *SCR1* gene, its termination activity was compromised, both *in vitro* and *in vivo*. Even the T₆ sequence, acting as a strong terminator in tRNA gene contexts, was unexpectedly weak within the *SNR52* transcription unit, where it naturally occurs. The observed sequence context effects reflect intrinsic recognition properties of Pol III, because they were still observed in a simplified *in vitro* transcription system only consisting of purified RNA polymerase and template DNA. Our findings strengthen the notion that termination signal recognition by Pol III is influenced in a complex way by the region surrounding the T cluster, and suggest that readthrough transcription beyond T clusters might play a significant role in the biogenesis of class III gene products.

Eukaryotic RNA polymerase (Pol) III is unique among DNA-dependent RNA polymerases in recognizing a simple run of T residues on the coding strand as a termination signal, in the apparent absence of accessory factors. The minimal signal thought to be sufficient to provoke Pol III termination varies among different eukaryotes. T₄ suffices for termination by *Xenopus* and human Pol III (1,2), T₄/T₅ for *S. pombe* Pol III (3), and T₅/T₆ for *S. cerevisiae* Pol III (4). Pol III termination within T clusters is generally heterogeneous and progressive (5), and termination efficiency tends to increase with the length of the T run (4). Mechanistically, Pol III termination involves extensive pausing, dictated by the T cluster itself (5,6). Even during elongation, the addition of three UMP residues in succession is particularly slow (5). Pol III pausing at T clusters sets cycles of hydrolytic RNA chain retraction (7). Both pausing and the associated hydrolytic RNA cleavage are affected by mutations in the C160, C128 and C11 subunits of yeast Pol III (7-9). In the case of C128 and C11, the same mutations have also been shown to affect the termination properties of Pol III, thus suggesting a functional interplay between hydrolytic RNA cleavage and transcription termination (9,10). The termination-prone character of Pol III is consistent with class III genes being very short, a fact that reduces the probability of unwanted termination signals within the coding regions. At the same time, the high efficiency of Pol III termination, by favoring
polymerase recycling and transcription reinitiation, is likely to contribute in a crucial way to the high level of transcript production typical of Pol III (11-13).

The facility of Pol III termination poses questions that have not yet been addressed exhaustively. One of them relates to the mechanism allowing for easy termination by Pol III. Pol I and Pol II transcription units very often contain long T clusters, yet these two RNA polymerases do not terminate at these sequences, even though T clusters are thought to generally induce RNA polymerase pausing. On which molecular features thus resides the distinctive ability of Pol III to terminate efficiently at short T clusters? At least part of the Pol III termination proneness has been attributed to the presence in this enzyme of the Rpc11p subunit, which is endowed with RNA cleavage stimulating activity (9). Such a feature, however, seems not to be unique to Pol III, as Pol I and Pol II both contain subunits that share sequence homology with Rpc11p and might similarly be involved in termination (14). A recent study has further suggested that the termination-prone character of Pol III may also come from a marked propensity of the enzyme towards RNA release, such that every T-rich sequence at which Pol III pauses sufficiently long time would become a termination site (15). Such a termination propensity take us to another important question, arising from the observation that Pol III-transcribed genes often contain internal stretches of T residues that would be predicted to provoke transcription termination. Is intragenic termination impaired in such cases, and by which mechanisms? A previously characterized example related to this issue is that of a tRNA Lys gene from X. laevis, in which two T4 tracts, one located at a canonical downstream position, the other one within the gene itself, determine transcription termination to different extents, with the internal T4 cluster behaving as a much weaker terminator than the external one (16). The idea that the sequence context can generally influence the efficiency of T clusters first emerged from early studies of Pol III termination (1), and was confirmed and extended by later studies of transcription termination on the adenovirus VA RNA genes (17,18). The surrounding sequences seem to influence Pol III termination especially at short T clusters. In Xenopus, for example, only T4 terminators display a context-dependent strength, while clusters of five or more T residues are highly efficient as terminators independently from the context (1). The few documented cases of surrounding sequence effects can hardly be accommodated into general rules of sequence specificity, because the same flanking nucleotides were found to produce different (or even opposite) effects on the terminators of different class III templates (1,16,18). Further adding to the complexity of the sequence requirements for Pol III termination, several observations also suggested the existence of Pol III termination signals that do not conform to the general “T-cluster” rule (18-20).

In this study, we show that termination at oligo(dT) tracts by S. cerevisiae Pol III can be influenced by the flanking sequences to a previously unsuspected extent, and we address the sequence requirements and molecular mechanisms of such effects.

**Experimental procedures**

**Statistical analysis of tDNA 3'-flanking regions** - Coordinates for the tDNA downstream regions were predicted directly from the genomic sequences of S. cerevisiae, S. pombe, M. musculus and H. sapiens by using Pol3scan (21) and tRNAscan-SE (22). Predicted tRNA genes were considered for the analysis if the tRNA boundaries and anticodon type were found to be identical by the two methods. Although this criterion does not guarantee the identification of all the genomic tDNAs, it strongly limits the occurrence of false positive results. The portions corresponding to the 100 nucleotides immediately downstream to the mature tRNA coding sequence were extracted as FASTA files and used for the analysis of terminator signals. A Perl script was designed for statistical analysis of T-runs of arbitrary length, making use of the BioPerl library (23).

**DNA templates** - For tRNA gene nomenclature, we refer to MIPS (Munich Information Center for Protein Sequences (http://mips.gsf.de/proj/yeast/rna/trna.html)). The P(AGG)CR, N(GTT)CR, N(GTT)KL, N(GTT)OL tDNAs have been described (24). The V(AAC)MR2 tDNA is carried by the previously described pY 7 plasmid (25). The Saccharomyces cerevisiae tDNA L(CAA)LR2 was PCR-amplified from yeast genomic DNA (strain S288C) by using the high fidelty Deep Vent DNA polymerase (New England Biolabs)
and the oligonucleotide primers L_fw (5'-GCAAATAAACGTTGGAAATTGGC) and L_rev (5'-GGTAGGAAAAAAGATGCTGCAC). The tDNAs S(AGA)EL and M(CAT)E were amplified by using the Pfu Turbo DNA polymerase (Stratagene) and the following pairs of oligonucleotide primers: S_fw (5'-GGGACTCTCAGAACGGGGG) and S_rev (5'-CGAGTTTTTACCATTAGGAAAG); M_fw (5'-GCGACATGGAGAGATAGAGATAG) and M_rev (5'-CGCAGAAGTTAAAGCTTATAGTC). The 3'-mutant forms of N(GTT)CR and L(CAA)LR2 were obtained by PCR using the cloned tDNAs as templates and the appropriate mutagenic reverse primers. All the amplification products were inserted into pUC-derived plasmid vectors and sequence-verified by dyeoxy chain termination sequencing. The SCR1Δ_T7 construct is the previously described 3'Δ+90 variant of SCR1 (26). For in vivo analysis, this SCR1 gene variant was subcloned into the YEp352 shuttle vector. The SCR1Δ_3'L template was obtained using the cloned wild type SCR1 gene as a template, the oligonucleotide 5'-TGATCAACTTAGCCAGGACATCC as a forward primer and the oligonucleotide 5'AAAAAAAAAACTTCAAAATTTTCGGAGGCAAAAACGTGCAATCCGG as a reverse primer. The amplified fragment, corresponding to the fusion of the first 90 bp of the SCR1 gene with the T5 terminator plus 20 bp of 3'-flank of L(CAA)LR2 followed by a T10 terminator, was cloned into the YEp352 vector. The 5'U6-L(CAA)LR2 and 5'U6-L_3'N templates were created by PCR using the corresponding cloned constructs as templates and the forward primer 5' TTTCGGCTACTATAAATAATGTTTTTTC GGAGCTACTCAAAATTTACAGCTGTGTGT TG, carrying the 5'-flanking sequence of the SNR6 gene of S.cerevisiae immediately followed by a SacI site (underlined). The amplified fragments were inserted into a modified pNEB193 plasmid whose polylinker SacI site was disrupted. The 5'U6-SCR1Δ_3'L construct was generated by PCR using the oligonucleotide 5'- TTTCGGCTACTATAAATAATGTTTTTTC GGAGCTACTCAAAATTTACAGCTGTGTGT TG, carrying the 5' sequence of the SNR6 gene, as a forward primer. In a different construct, the SacI restriction site was placed 5 bp upstream of SCR1Δ_3'L; the SCR1Δ_3'L variants were both inserted into the modified pNEB193. The SNR52 transcription unit was PCR-amplified from yeast genomic DNA (strain S288C) by using Pfu Turbo DNA polymerase and the following oligonucleotide primers: SNR52_fw (5'-TGATCAACTTAGCCAGGACATCC), SNR52_rev (5'-GGTAGGAAAAAAGATGCTGCAC), and SNR52_mutT mutant was constructed by recombinant PCR (27). Two overlapping PCR primary products were generated using the SNR52_fw oligonucleotide in combination with the mutT_rev primer (5'-GTAGGGTGAAACGATGCCGG). The amplified fragment, corresponding to the fusion of the first 90 bp of the SCR1 gene with the T5 terminator plus 20 bp of 3'-flank of L(CAA)LR2 followed by a T10 terminator, was cloned into the YEp352 vector. The 5'U6_SNR52 variant of SNR52, carrying the 5'-flanking sequence of SNR6, was generated by PCR using cloned wt SNR52 as a template and the oligonucleotide 5'-TTTCGGCTACTATAAATAATGTTTTTTC GGAGC as a forward primer. All the SNR52 variants were cloned into the pNEB193 plasmid vector.

In vitro transcription analyses - In vitro transcription reaction conditions, and procedures for RNA purification and analysis, were essentially as described (13,28). Reactions contained 40 fmol of plasmid-borne class III template, and the following amounts of transcription proteins: 150 ng of TFIIIC purified up to the DEAE-Sephadex A-25 step (29); 40 ng of pure, recombinant TATA box binding protein (TBP) and 80 ng of recombinant Brf1, both purified from overexpressing E. coli cells (29); 0.5 µg of B” fraction partially purified, up to the Bio-Rex 70 chromatography step, from chromatin pellets generated during yeast nuclear extract preparation (30). The last three components reconstitute a highly active TFIIIB factor. Unless otherwise indicated, the ATP, CTP and GTP concentration was 500 µM, UTP concentration was 250 µM. Transcripts were radiolabeled using [α-32P]UTP. Template DNA was first incubated with TFIIIC and TFIIIB for
20 min at 20°C, then Pol III (10 ng) and NTPs were added and transcription was allowed to proceed for 20 min at 20°C. In Figures 7A and 8B, transcription complexes were assembled either in the presence or in the absence of TFIIIC, using increased amounts of the different components of TFIIIB: 400 ng of TBP, 240 ng of Brf1 and either 0.7 μg of B” fraction (Figure 8B) or 20 ng of recombinant yeast Bdp1 (13) (Figure 7A). For the factor-less transcription experiment in figure 7B, 3’-overhanged restriction fragments were obtained by digesting the above described SacI-site containing templates with SacI, cutting 5 bp upstream of the natural transcription start site, and either BamHI (for L(CAA)LR2) or NdeI (for L_3’N and SCR1Δ 3’L), cutting downstream of the cloned insert in the plasmid polylinker. Restriction fragments were gel-purified, treated once with phenol:chloroform and ethanol-precipitated prior to use in transcription assays. General transcription conditions were the same as in factor-directed transcription. The 3’-overhanged fragment (30 ng) was first incubated with 20 ng of purified Pol III and 400μM C³P dinucleotide primer in transcription buffer containing 160 μg/ml BSA for 15’ at 20°C, in a total volume of 25µl. NTPs and [α-³²P]UTP (10 μCi) were then added and transcription was allowed to proceed for 30 min at 20°C. In all transcription experiments, radioactive transcripts were quantified by phosphorimaging. For termination efficiency measurements, the signals corresponding to RNAs of different length were normalized for the number of incorporated U residues.

In vivo RNA analysis - Yeast cells (YPH500 strain) were transformed with the different YEps352-SCR1Δ constructs by the lithium acetate procedure (31) and the resulting transformants were selected for uracil auxotrophy. Total RNA was prepared according to a previously described procedure (32). RNA samples (10 μg) were electrophoresed on 6% polyacrylamide, 7 M urea gels and transferred to a GeneScreen Plus membrane (Perkin Elmer) which was then probed with a 5’-labeled oligonucleotide (5’-CTGGCCGAGGAACAAATCCTTCTCCTCGCGC) complementary to the coding region of SCR1 between position +43 and +73. Hybridization was carried out overnight at 28°C in 5X SSC solution, 5X Denhardt’s solution, 0,1 mg/ml denatured salmon sperm DNA, 0,5% (w/v) SDS, followed by one short washing in 2X SSC solution containing 0,1% SDS and two short washings in 1X SSC solution containing 0,1% SDS. Hybridization products were visualized and quantified by phosphorimaging.

Results

Oligo(dT) termination signals in different eukaryotic genomes - The flanking regions of eukaryotic tRNA genes present conserved sequence patterns that diverge considerably in going from yeast to mammalian genomes (24). As a further element of divergence, yeast and human RNA polymerase III enzymes respond differently to oligo(dT) terminators (3). To gain insight into Pol III termination signals on a genome-wide scale, we thus decided to analyze and compare the tDNA transcription terminators in two yeast (S. cerevisiae, S. pombe) and two mammalian (M. musculus, H. sapiens) genomes. Using the complete tRNA gene inventories from these genomes, we initially searched for the presence of runs of 4 or more T residues within the first 100 bp of the 3’-flanking region, because T₄ is the shortest known oligo(dT) tract causing Pol III termination (33). In S. cerevisiae and S. pombe, T₄ was never found as the sole potential termination signal: a run of 5 or more T residues was always present, and it always started within the first 40 bp following the end of the mature tRNA coding sequence. This was taken as an indication that runs of less than 5 Ts are not used as Pol III terminators in yeast, and that Pol III termination signals appear within the first 40 bp of 3’-flanking sequence. In contrast, many mammalian tDNAs had a run of 4 Ts as the sole potential termination signal in the same region, in agreement with the reported ability of human Pol III to terminate efficiently at T₄, and with the observation that yeast Pol III requires longer T stretches than vertebrate Pol III in order to terminate efficiently (3,33). Again, most termination signals occurred within 40 bp of 3’-flanking region. Therefore, we considered T₃ and T₄ as the minimal termination signals for yeast and mammalian tDNA transcription, respectively. By limiting further analysis to the first 40 bp of 3’-flanking sequence, we then selected, from each tDNA set, the tDNAs having a single T run within that region. Such “single-terminator” tDNAs represented 56%, 47%, 69% and 67% of all tDNAs in S. cerevisiae, S. pombe, H. sapiens and M. musculus, respectively. We then calculated, for the subsets of “single-
terminator” tDNAs from each genome, the frequency distribution of T run length. The aim of such analysis was to establish whether the T run length in the different tDNA sets correlates with lineage-specific differences in termination signal recognition by Pol III. The tDNAs with more than one termination signal within the first 40 bp of 3’ flank were excluded from analysis, because the presence of a potential “back up” terminator could have reduced the selective pressure on the upstream terminator. The results of this analysis are shown in Figure 1. In S. cerevisiae and S. pombe, the most frequent T run lengths are 6 and 7, and (as already mentioned) there is no single terminator shorter than T5. Remarkably, the tDNA terminators in fission yeast tend to be ~1 nt shorter than in S. cerevisiae, in agreement with a previous analysis showing that S. cerevisiae Pol III requires slightly longer T runs than the fission yeast enzyme in order to terminate efficiently (3). The T-run length distribution in mammals is completely different: T4 is the most frequent tDNA terminator, and terminators longer than T4 are rare. Again, this finding is in agreement with the reported ability of human Pol III (and, more generally, of vertebrate Pols III) to terminate efficiently at T4 sequences (1,3,18,33).

**Variable strength of the T₅ terminator in S. cerevisiae tDNAs** - Of the 148 “single-terminator” tDNAs of S. cerevisiae, 22 bear a simple run of 5 Ts as a termination signal. For such tDNAs, the T₅ sequence is thus expected to be endowed with the ability to induce efficient Pol III termination. On the other hand, previous studies have shown that 5 consecutive T residues in the 3’-flanking sequence of yeast tDNAs are only partially effective in inducing Pol III transcription termination (3,4). To better define the termination properties of the T₅ sequence, we initially analyzed transcription termination on 8 yeast tRNA genes all displaying T₅ as the first termination signal encountered in the 3’-flanking region. These genes are listed in Figure 2A, together with the features of their 3’ flanks. Two of them (#1 and #2) are classified as “single-terminator” tDNAs. In the other tRNA genes, T₅ is followed by a back up terminator located 3-20 bp downstream. The presence of a downstream T run allows to easily monitor polymerases reading through the T₅ terminator by their production of longer transcripts terminated at the downstream T cluster. This is also true in the case of tDNA #1 (L(CAA)LR2), as a back-up T run is present starting at 66 bp downstream of T₅. In the case of P(AGG)CR, where no back-up T run is present, the tDNA-containing plasmid was linearized 121 bp downstream of T₅, so as to be able to reveal reading-through polymerases by their production of longer, run-off transcripts. All these templates were used to program in vitro transcription reactions in a reconstituted system from S. cerevisiae (see Experimental Procedures).

Transcription was performed in the presence of either a low UTP concentration (25 µM, left panel of Figure 2B) or a ten-fold higher UTP concentration (right panel of Figure 2B) closer to the in vivo UTP concentration, estimated to be in the millimolar range (34,35). Figure 2B shows that the strength of T₅ as a terminator varies considerably in the different contexts, and that T₅ readthrough by Pol III is in some cases strongly favored at the higher UTP concentration (cf. for example lanes 3, 4 of the left panel with the same lanes of the right panel), i.e. under conditions that increase the polymerization rate and thus decrease the dwell time of the enzyme at T₅. For six out of the eight genes the T₅ element acted as a strong terminator (lanes 1, 2 and 5-8). In contrast, for two tDNAs (lanes 3 and 4) a large fraction of polymerases read through the T₅ element and terminated at the back up terminator. The two T₅ elements behaving as weak terminators are both immediately followed by the sequence CT and, a few base pairs downstream, by a longer T run acting as a back up terminator (tDNA #3 and #4 in Figure 2A). In contrast, the six T₅ elements acting as strong terminators are all immediately followed by A or G, with the exception of M(CAT)E (tDNA #5). The T₅ element of M(CAT)E is immediately followed by C, and is characterized by a slightly lower strength as a terminator (cf. lane 5 with lanes 1, 2, 6, 7, 8 in the right panel of Figure 2B). It is interesting to note that T₅ is flanked by almost identical upstream sequences in the case of N(GTT)CR (tDNA #3), N(GTT)KL (tDNA #6) and N(GTT)OL (tDNA #7). Therefore, the remarkable difference in terminator strength between N(GTT)CR (lanes 3) and the other two tRNAₐ₃m genes (lanes 6 and 7) can reasonably be attributed to the downstream sequence context.

**Influence of the downstream sequence on Pol III termination at T₅** - To investigate in more detail the downstream sequence effect on T₅ terminator strength, we focused on two of the tDNAs tested in the previous experiment: L(CAA)LR2 (tDNA #1), whose T₅ is one of the strongest terminators,
and N(GTT)CR (tDNA #3), whose T₅ is the weakest terminator observed in Figure 2 and is flanked by a back up terminator 7 bp downstream. We initially constructed and tested two hybrid templates (Figure 3A): the L₃’N template, in which the sequence downstream of the L(CAA)LR2 T₅ terminator was replaced by the CT₄CT₁₃ sequence naturally present downstream of T₅ in N(GTT)CR, and the N₃’L template, in which the sequence downstream of the N(GTT)CR T₅ element was replaced by 20 bp of the sequence naturally present downstream of the L(CAA)LR2 T₅ terminator, followed by an artificially placed T₁₀ back-up terminator. In the L₃’N context, the T₅ terminator of L(CAA)LR2 turned out to be remarkably weakened, displaying (at 250 µM UTP) a termination efficiency of 55% as compared to 98% in the case of the wild type L(CAA)LR2 gene (Figure 3B, cf. lanes 1 and 2). On the other hand, when N(GTT)CR with its T₅ element was fused to the 3’-flanking sequence derived from L(CAA)LR2, the strength of T₅ as a terminator was greatly increased (Figure 3B, cf. lanes 4 and 5). There are two possible explanations for the above observations. The T₅ element might be intrinsically weak as a terminator, and the particular downstream context in L(CAA)LR2 would potentiate its termination proficiency; in this case, T₅ weakening in L₃’N would be simply due to the removal, and T₅ strengthening in N₃’L to the insertion, of a terminator-strengthening sequence. Alternatively, the CT₄CT₁₃ sequence naturally present 3’ to the T₅ element in N(GTT)CR might directly act as (or contain) a terminator-weakening element. According to the second hypothesis, the replacement of the sequence downstream of T₅ in N(GTT)CR with an unrelated sequence should increase the T₅ strength as a terminator, while the replacement of the sequence downstream of T₅ in L(CAA)LR2 with an unrelated sequence should not decrease the T₅ terminator efficiency. In the experiment in Figure 3B, the termination properties of the unmodified L(CAA)LR2 and of the L₃’N construct were compared with those of a L(CAA)LR2 derivative (L₃’∆3’) in which the 3’-flanking sequence downstream of T₅ was replaced by a plasmid sequence (lane 3). Similarly, the termination properties of unmodified N(GTT)CR and of the N₃’L construct were compared with those of a N(GTT)CR derivative (N₃’∆3’) in which the T₅ 3’ flank was replaced by the same plasmid sequence as in L₃’∆3’ (lane 6). Termination at T₅ appeared to be unaffected in the L₃’∆3’ construct with respect to unmodified L(CAA)LR2 (cf. lanes 1 and 3) while the strength of the N(GTT)CR T₅ element was greatly increased in the N₃’∆3’ construct (cf. lanes 4 and 6). (Since no rescue terminator is present downstream of T₅ in the L₃’∆3’ and N₃’∆3’ templates, they were linearized with Bam HI at a position 16 bp downstream of the T₅ sequence). The CT₄CT₁₃ sequence thus behaves as (or appears to contain) a terminator-weakening element. This conclusion is further supported by the observation that T₅ is weak in only two of the eight tDNAs tested in the experiment in Figure 2 (N(GTT)CR and S(AGA)EL), and that in both cases T₅ is followed by similar CT-containing sequences (CT₄CT₁₃ in the case of N(GTT)CR, CTCT₆C in the case of S(AGA)EL).

Sequence and positional requirements for T₅ terminator weakening - To better understand the T₅ terminator weakening effect we concentrated on the L₃’N chimeric template, whose downstream weakening sequence, CT₄CT₁₃, was subjected to mutational analysis. In particular, mutants were constructed in which the CT₄C sequence was variously replaced, whereas the downstream T₁₃ tract was left unvaried (or shortened to T₁₀) and used as a back-up terminator. All the mutant constructs, schematically illustrated in Figure 4A, were tested in an in vitro reconstituted system in at least three independent experiments. The results are shown in Figure 4B. Pol III transcriptional readthrough at T₅ was first calculated on the basis of the ratio between T₅-terminated and T₁₃/T₁₀-terminated transcripts. In each experiment, the extent of T₅ terminator readthrough observed with the L₃’N template (lane 2) was arbitrarily set to 100, and the effect of the mutations was evaluated as relative T₅ readthrough with respect to L₃’N. When the first three nucleotides downstream of T₅ (CTT) were changed to GCC, so as to render the 6 base pairs downstream of T₅ identical to the sequence found in the wild type L(CAA)LR2 tDNA, the weakening effect was strongly diminished (~5-fold) with respect to L₃’N (cf. lanes 2 and 4). Since the CTT to GCC substitution, which increases the predicted stability of the DNA double helix immediately downstream of T₅, strongly affected its weakening potential, we wondered whether replacing the downstream sequence with other sequences characterized by a similar (or even lower) DNA duplex stability would preserve (or
increase) the weakening effect, and thus T₄ readthrough. To address this point, the T₄ stretch in CT₄C was replaced by TATA, ATAT or A₄. As shown in Figure 4B, lanes 6-8, and in the bar plot above the gel, such substitutions led to a partial loss of the weakening effect: a 40-50% loss in the case of A₄ and ATAT, only a 20% loss in the case of TATA. The prediction of DNA duplex stability on the basis on nearest-neighbor thermodynamics gives A₄ = T₄ > ATAT > TATA (36). Therefore, among the three substitutions, the one causing the strongest readthrough (TATA) is also the one displaying the lesser DNA duplex stability. However, despite its higher predicted stability, the original T₄ sequence causes more readthrough than TATA. We conclude that a low DNA duplex stability in the region immediately downstream of T₅ is not sufficient for full T₅ terminator weakening, even if it somehow contributes to the weakening effect. This conclusion is also supported by the comparison of the termination properties of tDNAs #3 and #7 in Figure 2. The T₅ terminator of tDNA #7 is much stronger than the T₅ terminator of tDNA #3, yet the predicted stability of the immediately downstream 6-bp sequence (CT₄C for tDNA #3, GAATAC for tDNA #7) is very similar (36). With another series of constructs (templates #5, #9 and #10 in Figure 4) we then analyzed the positional requirements for T₅ terminator weakening. In all of these constructs, the T₁₃ element was replaced by T₁₀, a sequence which works as efficiently as T₁₃ as a downstream terminator, and does not interfere with the T₅ weakening effect (data not shown). In construct #5, the distance between the T₅ and T₁₀ stretches, that are separated by only 1 bp in the L_3’N template (lane 2), was increased to 11 bp. As shown in Figure 4B, T₅ terminator weakening in this context was very similar to that observed with L_3’N (cf. lanes 2 and 5). The CT₄C sequence thus appears to act as an autonomous, termination-weakening element. In contrast, the weakening effect was found to be compromised in constructs in which the distance between T₅ and the CT₄C sequence was increased by 3 bp (construct #10, lane 10) or even by only 1 bp (construct #9, lane 9). The terminator weakening effect exerted by the CT₄C sequence thus seems to depend in a critical way on its distance from the T₅ terminator. Having restricted the terminator-weakening function to the CT₄C sequence, we constructed other mutants (constructs #11-15 in Figure 4A) in order to identify the minimal sequence requirements for the terminator weakening effect. As shown in lane 13, the C residue separating the T₅ stretch from the downstream T₁₀ sequence could be mutated without any loss of terminator weakening (on the contrary, a 50% increase in T₅ readthrough with respect to L_3’N was reproducibly observed with this mutant; cf. lanes 2 and 13). With templates #14 and #15, in which the length of the T₄ stretch was progressively reduced, the weakening effect was maintained (lanes 14 and 15). The mutation of the C residue, immediately following the T₅ element, to A reproducibly reduced the readthrough efficiency by ~65% with respect to L_3’N (cf. lanes 2 and 11). The mutation of the same residue to G produced a more modest, but still significant effect, reducing T₅ readthrough by ~40% with respect to L_3’N (cf. lanes 2 and 12). A C residue immediately downstream of T₅ is thus required for full weakening efficiency. The results of this mutational analysis, together with the data in Figure 2, suggest that a CT dinucleotide immediately downstream of a T₅ terminator represents a minimal sequence element capable of producing a significant terminator weakening effect. That CT alone is sufficient to induce readthrough is especially evident from the termination properties of tDNA #4 in Figure 2 and template #8 in Figure 4. This weakening element, however, appears to be only effective on the T₅ terminator. When the termination signal length was increased to T₁₀, the weakening effect was lost (lane 16 in Figure 4B).

**Mechanism of T₅ terminator weakening by downstream sequence** - The oligo(dT) tracts acting as Pol III terminators have the ability to induce both RNA polymerase pausing and transcript release. These two features are functionally distinguishable, and are characterized by different sequence requirements (6,15). Weakening of the T₅ terminator by a CT sequence placed immediately downstream may involve an impairment of both (or only one) of these abilities. To gain insight into the mechanism of T₅ terminator weakening, we first analyzed termination on the L_3’N template (construct #2 in Figure 4A) and on the L_3’GT₄ template (template #12 of figure 4A, in which the C immediately downstream of T₅ is replaced by G) in the presence of a standard (500 μM) and a 10-fold lower concentration of CTP. Lowering CTP concentration is expected to increase the dwell time of Pol III across the T₅ sequence in L_3’N, but not in L_3’GT₄, by reducing the rate
of incorporation of the first nucleotide downstream of T₅. In the same way, lowering the GTP concentration should increase the dwell time of Pol III onto the T₅ element in L₃’GT₄α, but not in L₃’N. As shown in Figure 5, in the presence of 50 μM CTP (lane 5) the T₅ terminator in L₃’N turned out to be significantly stronger than with 500 μM CTP (lane 2), with a ~3-fold reduction in the fraction of reading-through polymerases (from 45% in lane 2 to 14% in lane 5). Lowering the GTP concentration had instead no effect on T₅ readthrough with this template (cf. lanes 2 and 8). With the L₃’GT₄α template, lowering the CTP concentration did not influence the extent of T₅ readthrough, while the fraction of reading-through polymerases was slightly (but reproducibly) decreased at low GTP concentration (from 22% in lane 3 to 17% in lane 9). T₅ terminator weakening can thus be specifically counteracted by reaction conditions that favor polymerase pausing on T₅.

Effect of the upstream sequence context on T₅ terminator efficiency - Altogether the above data show that termination efficiency at T₅ can be strongly influenced by the downstream sequence context, in a manner that is independent from the specific tRNA coding sequence preceding the T₅ element. To further test for possible effects of the upstream flanking sequence on T₅-dependent termination, we generated a construct (SCR1₃’L) in which the L(CAA)LR2 T₅ terminator, plus 20 bp of its natural 3′-flanking sequence followed by T₁₀, were fused to the first 90 bp of the SCR1 gene, coding for the S. cerevisiae 7SL RNA. Apart from the presence of A- and B-blocks allowing for efficient in vitro transcription by the Pol III system (26), this portion of the SCR1 gene is essentially unrelated to tRNA coding sequences. As shown in Figure 6A, lane 1, the insertion of a T₇ terminator at position +90 of SCR1 results in a template (SCR1₃’T₇) whose transcription is efficiently terminated at T₇, thus generating 90 nt-long transcripts; no readthrough transcription products were observed when this template was linearized 25 bp downstream of T₇ (data not shown). Surprisingly, the L(CAA)LR2-derived T₅ element plus 3′ flank, that produced a 97% termination efficiency in the case of the original template (see for example Figure 2, lane 1), behaved as a poor terminator in the SCR1₃’L construct, allowing ~40% of the polymerases to read through and terminate at the downstream T₁₀ terminator in the presence of 250 μM UTP (Figure 6A, lane 2). Readthrough was significantly lesser in the presence of 25 μM UTP (lane 4) and almost absent with 2.5 μM UTP (lane 6), thus suggesting that the SCR1-derived upstream sequence reduces termination at T₅ by affecting Pol III pausing. Transcription termination with the SCR1₃’L construct could also be monitored in vivo, because the 20 bp distance between the T₅ element and the downstream T₁₀ terminator allows for the production of transcripts that differ from each other in size significantly, and that are characterized by comparable in vivo stabilities. The SCR1₃’T₇ and SCR1₃’L templates were inserted into the high copy number YEp352 vector and transformed into the YPH500 S. cerevisiae strain. The RNAs derived from logarithmically growing cells were then subjected to Northern blot analysis with an SCR1-specific antisense oligonucleotide. As shown in Figure 6B (lane 2) 55% of the plasmid-encoded, SCR1-derived transcripts were terminated at T₅, while 45% were 20-nt longer and derived from T₅ readthrough and termination at the downstream T₁₀ sequence. By comparison, the T₇ terminator in the SCR1₃’T₇ construct appeared to be fully efficient in vivo (lane 1).

Distinguishing between weak and strong T₅ terminator sequences is an intrinsic property of Pol III - The observed sequence context effects on Pol III termination at T₅ might in principle be mediated by TFIIIC, a transcription factor that usually covers a large part of the transcription unit, extending up to the terminator region of tRNA genes (37,38). TFIIIC-associated activities have previously been proposed to influence Pol III termination in humans (39), and yeast TFIIIC has recently been shown to facilitate transcription reinitiation by Pol III on long transcription units (13). The role of TFIIIC is thus not restricted to preinitiation complex assembly. To test whether TFIIIC influences termination at T₅ in different sequence contexts, some of the templates in which the T₅ terminator was weakened were modified so as to allow for their TFIIIC-independent transcription. To this end, the transcribed portions of L(CAA)LR2, L₃’N and SCR1₃’L were fused to the 5′-flanking region of the S. cerevisiae SNR6 gene, which is known to support TATA box-mediated TFIIIB assembly and transcription initiation in the absence of TFIIIC (13,40). As shown in Figure 7A, a
pronounced weakening of T₅ by both the 3’N downstream context and the SCR1Δ upstream context was observed in the absence of TFIIIC (cf. lanes 5, 6 with lane 4), thus demonstrating that the weakening effects do not depend on this factor. A comparison of the termination efficiencies in the presence (lanes 1-3) or absence (lanes 4-6) of TFIIIC rather suggests that TFIIIC somehow facilitated T₅ termination signal recognition by Pol III, an effect that is especially evident with the 5’U6-L_3’N template (cf. lanes 2 and 5 in Figure 7A). To test in a more direct way whether distinguishing between weak and strong T₅ termination signals is an intrinsic property of Pol III, we exploited the reported ability of Pol III to initiate transcription on linear templates containing a 3’-overhanging end (41). Linear, 3’-overhanded versions of L(CAA)LR2, L_3’N and SCR1Δ_3’L templates were prepared as described in the Experimental Procedures section and transcribed in vitro with purified RNA polymerase III in the absence of any transcription factor. As shown in Figure 7B, and in agreement with a previous study (41), purified Pol III specifically and efficiently transcribed these templates. Transcription initiation was made to occur at the 3’-overhanging end generated by SacI cleavage, at a position 5 bp upstream of the natural transcription start site. Pol III terminated transcription at the same terminator region present in the original templates, in which a T₅ element of variable strength is followed by a back-up terminator (placed 67 bp, 7 bp and 21 bp downstream of T₅ in L(CAA)LR2, L_3’N and SCR1Δ_3’L respectively). A small percentage of transcription complexes (10% at most) failed to recognize both the T₅ and back-up terminators thus giving rise to run-off products (the DNA fragments used as templates ended 99, 238 and 249 bp downstream of T₅ in L(CAA)LR2, L_3’N and SCR1Δ_3’L respectively). Such a small amount of run-off transcripts probably derives from transcription complexes unable to displace the RNA from RNA:DNA hybrids, a reported artifact of initiating transcription at 3’-overhanded or dC-tailed templates (6,41). Run-off transcripts were thus not considered for quantification purposes. The T₅ signal in the wild type context of L(CAA)LR2 acted as a rather strong terminator, both at 25 µM and at 250 µM UTP, with only 19% of transcription complexes reading through and terminating at the T₅ back up terminator (Figure 7B, lanes 1 and 4). In contrast, the T₅ termination signal was remarkably weakened in the L_3’N and SCR1Δ_3’L contexts, both at 25 µM UTP (lanes 2, 3) and at 250 µM UTP (lanes 5, 6). The weakening effect was dramatic in the case of SCR1Δ_3’L, for which no transcripts terminated at T₅ could be detected at 250 µM UTP (lane 6). Weakening of T₅ by both the upstream and the downstream sequence contexts thus clearly reflect intrinsic recognition properties of Pol III. By comparing the extents of T₅ weakening observed at 250 µM UTP in the absence of transcription factors with those observed under the same conditions with a complete transcription system (see for example Figure 5, lane 2 and Figure 6, lane 2) we note that the weakening effect is significantly enhanced in the factor-free system. Such a T₅ readthrough enhancement can at least in part be attributed to the absence of TFIIIC (see above the comments to Figure 7A).

Antitermination within a natural Pol III transcription unit - Recent studies of the genome-wide localization of the Pol III transcription machinery have identified the snoRNA encoding SNR52 gene as a new class III gene (42-44). Northern blot and primer extension analyses suggested that the primary transcript of SNR52 is a ~250 nt precursor RNA from which a leader sequence of 160 nt is cleaved to generate the ~90 nt mature snoRNA product, and sequence analysis revealed the presence of putative A- and B-block elements within the leader sequence (42,43). An unusual feature of this sequence, however, is that a run of 6 Ts is present within the transcribed region, a few bp downstream of the putative A-block. The T₆ sequence is a highly efficient termination signal for yeast Pol III, as illustrated by the results shown in Figure 4B (lane 16) and by the results of in vitro transcription experiments with several tDNAs bearing a T₆ terminator (data not shown). As shown in Figure 8 (lane 1), in vitro transcription of SNR52, in the presence of 25 µM UTP, produced two main transcripts. The longer transcript was identified as the SNR52 primary transcript on the basis of its size (250 nt). The shorter RNA had the size expected for a transcript terminated at the intragenic T₆ element. Indeed, transcription of a mutant SNR52 template, in which T₆ was changed to TTCGTT, produced higher levels of the longer transcript, while the shorter transcript disappeared (cf. lanes 1 and 2). Quantification of the transcripts in lane 1, corrected for the number of incorporated U
residues, led to estimate that as much as 70% of the polymerases read through the T₆ element in SNR52. T₆ thus behaves as an unusually weak terminator in the SNR52 context, even under conditions (low UTP concentration) that favour termination at T stretches. The inactive T₆ element of SNR52 is located just downstream of a putative A-block promoter element within the transcribed region. Since TFIIIC is expected to contact such region during transcription, this factor might be required in order for Pol III to read through the internal T₆. To test this possibility, the SNR52 transcription unit was fused with the 5′-flanking region of the S. cerevisiae SNR6 gene, to allow for TFIIIC-independent transcription of the resulting chimeric template. As shown in Figure 8B, the presence or absence of TFIIIC on SNR52 had no influence on the termination behaviour of Pol III, characterized by highly inefficient termination at the internal T₆ element at 250 µM UTP (cf. lanes 2 and 4).

Discussion

This work demonstrates that the recognition of oligo(dT) termination signals by S. cerevisiae RNA polymerase III can be strongly influenced by the sequence context in which the T stretch is embedded. Such influence becomes evident with the shortest termination signals encountered in yeast class III genes, namely T₃ and T₆, that together characterize ~40% of the yeast tDNAs possessing a single T run as a termination signal (see Figure 1). We have been able to distinguish between two types of sequence context effect: one, that we have more extensively addressed, is due to the few base pairs immediately downstream of T₃; the other was observed when a non-tDNA sequence was fused upstream of a strong terminator region (composed of T₃ plus a particular 3′ flank) derived from L(CAA)LR2 tDNA. An extreme example of sequence context effect was observed with the SNR52 transcription unit, in which a T₆ element placed between the A-block and the B-block promoter elements was barely recognized as a terminator by Pol III.

The main conclusion emerging from the study of the downstream sequence effect is that T₃, when associated with tRNA genes, is intrinsically strong as a terminator, because it induces highly efficient termination in most of the contexts analyzed; however, features of the downstream sequence exist that can significantly weaken the T₃ termination potential. A specific weakening element identified in this study seems to minimally consist of the CT dinucleotide placed immediately downstream of T₃ in the coding DNA strand. The tested natural tDNAs displaying such a feature were characterized by incomplete termination at T₃. The 3′ flank of N(GTT)CR, one of these tDNAs, could be transplanted 3′ to another tDNA, L(CAA)LR2, without losing its T₃ terminator weakening properties. By extensive mutagenesis, we were able to show that T₃ weakening absolutely requires a C residue immediately downstream of T₃, and is greatly favoured by the presence of at least one T just after the C. With this respect, it is interesting to note that, in an early study of yeast Pol III termination, termination at T₃ was found to be incomplete, both in vitro and in vivo, for a template in which T₃ was followed by CT (4). T₃ terminator weakening might not be an exclusive property of the CT element, and other short weakening sequences may exist. However, neither natural tDNA analysis nor extensive mutagenesis of the N(GTT)CR-derived weakening element could reveal any other sequence with higher or similar weakening activity. By which mechanism does CT downstream of T₃ induce terminator readthrough? Our data suggest that CT facilitates Pol III translocation by reducing its pause time at T₃. Indeed, readthrough tends to be lesser under conditions (such as low UTP or CTP concentrations) that increase the dwell time of Pol III across the T₃ tract. We also tested the sensitivity of T₃ terminator weakening to variations in KCl concentration (50-150 mM range) and Mg²⁺ ion concentration (3-10 mM range), which have the potential to influence the RNA release step of intrinsic bacterial termination (45), but we did not observe any significant variation in termination efficiency at T₃ either in the L(CAA)LR2 or in the L₃′N contexts (data not shown). Possible mechanistic explanations of the effect of downstream DNA on translocation include the stability of the duplex that must be melted, or NTP binding to a melted region of downstream DNA, which would facilitate translocation (46-49). Our data tend to exclude a simple correlation between T₃ terminator weakening by downstream DNA and the ease of duplex melting. For example, template #6 in Figure 4 displayed the same duplex stability as template #2 in the region immediately downstream of T₃ (CA₄ vs. CT₄), yet the weakening effect was ~2-fold lower with
CA₄ than with CT₄. Also, the introduction, in template #9 of Figure 4, of an A residue before CT₄ completely abolished the weakening effect of CT₄ without changing significantly the duplex stability of this region. Our data rather suggest that the CT element might increase the processivity of Pol III at the position immediately upstream, possibly by a mechanism involving the binding of the corresponding NTPs to an allosteric NTP binding site, as recently proposed to explain downstream DNA effects on elongation by both E. coli RNA polymerase (49) and yeast RNA polymerase II (48).

Having established that T₅, followed by the sequence naturally present in the L(CAA)L₇R2 tDNA, behaves as a strong terminator for Pol III, even when fused 3' to a completely different tDNA (see Figure 3), we were surprised to find that the same sequence becomes a much weaker terminator when fused 3' to an A- and B-block-containing segment of the SCR1 gene. Even more surprising was the finding that the T₆ sequence, generally producing highly efficient Pol III termination, was substantially inactive as a terminator in the context of the SNR52 gene, where it is naturally present between the A-block and B-block promoter elements preceding the snoRNA coding sequence. Although the sequence and positional requirements for both the SCR1 and the SNR52 effects were not investigated in detail, in both cases readthrough was found to be favored at higher UTP concentrations, thus indicating that these effects involve an alteration of Pol III pausing. The results of experiments in which transcription initiation and termination were made to occur in the absence of TFIIC further suggest that T₅/T₆ readthrough in these two contexts does not require TFIIC. Rather, in the absence of TFIIC readthrough tended to be more pronounced, an observation that might be explained by postulating that DNA-bound TFIIC slightly slows RNA chain elongation thus favouring termination at T₅. DNA-bound TFIIC has previously been shown to exert no significant effect on RNA chain elongation by Pol III in the sense direction of the SUP4 tRNA³ Tyr gene (41). It can not be excluded, however, that in specific sequence contexts TFIIC contributes to Pol III pausing and termination. In vitro transcription experiments conducted in the presence of purified Pol III and template DNA only, we showed that T₅ terminator weakening also occurs in the absence of any transcription factor, and thus reflects intrinsic recognition properties of Pol III, a conclusion in agreement with that of an early study of termination by Xenopus Pol III (2). The T₅ weakening effect was especially evident when the strong L(CAA)L₇R2 terminator region was placed downstream of an A- and B-block-containing fragment of the SCR1 gene. In this case, T₅ readthrough enhancement can only depend on particular features of the upstream DNA region. This region might influence the Pol III behaviour at T₅ through polymerase-DNA interactions, but it might also play an active role in the termination process through the corresponding RNA product, as is well known for the stem-loop coding portion of intrinsic bacterial terminators (50,51).

The lack of termination at T₆ within the SNR52 transcription unit is of evident biological significance, as the snoRNA coding portion of SNR52 is entirely located downstream of the T₆ element. Curiously such sequence, which is potentially detrimental to SNR52 gene transcription, is also present in the genomes of at least two other Saccharomyces species (S. paradoxus and S. mikatae (52)), thus indicating a possible relevant role in SNR52 expression. In principle, the T₆ element might influence the accessibility and/or transcriptional activity of SNR52 in vivo, or it might play a role in pre-snoRNA processing. Less evident is the biological role of T₅ terminator weakening by short downstream sequences like CT in the case of tRNA genes. As a result of T₅ readthrough, Pol III is expected to synthesize in vivo tRNA precursors with elongated 3’ trailers, that might in principle stabilize the pre-tRNA from 3’ exonucleolytic digestion. In organisms from yeast to humans, binding of La protein also protects the pre-tRNA 3’ trailer from exonucleolytic digestion (reviewed in(53)). La protein binds the UUUₐ₉₈ at the 3’ end of nascent Pol III transcripts and, by favoring the formation of correctly folded pre-tRNAs, it can affect pre-tRNA stability, processing, localization and charging (53-56). The 3’ trailer extension caused by Pol III readthrough might influence each of these processes either directly or by affecting La binding. In support of this view, it has recently been shown that an increase in 3’-terminal oligo(U) length of pre-tRNAs can influence association with La and tRNA maturation (57). The modulation of Pol III terminator readthrough might be of even higher significance in mammals. In the mouse and human genomes, T₄ is by far the most frequent termination signal for tRNA gene transcription, and in many cases it is
the sole potential termination signal encountered 3’ to the tDNA. Since runs of 4 Ts are often present within the internal transcribed region of tRNA genes, there must be features of the sequence context capable of strongly affecting Pol III termination at T₄. Such features, however, are not simply represented by conserved sequence elements flanking the T₄ terminator, as revealed by statistical analysis of such regions in mouse and human tDNA datasets⁴. Why did mammalian tDNA terminators evolve towards the shortest possible T runs? One possible answer is that, in the presence of weak T run terminators, more room is left for the modulation of termination by accessory factors, such as NF1, affecting transcription termination by human Pol III (39). Alternatively, the predominance of very short T runs downstream of mammalian tDNAs might reflect the importance of short 3’ polyuridylate tails for the post-transcriptional fate of mammalian pre-tRNAs.

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Footnotes

1 The abbreviations used are: BSA, bovine serum albumin; NTP, ribonucleoside triphosphate; PCR, polymerase chain reaction; Pol, RNA polymerase; SDS, sodium dodecyl sulfate; TF, transcription factor; wt, wild type.
The only exceptions were represented by dimeric tDNAs, in which the upstream tDNA unit has no termination signal (58,59).

The very low stability of 3’-extended derivatives with respect to 3’-matured products of tRNA genes, together with the presence in multiple copies of most tRNA genes (21), generally complicates the detection of in vivo termination products in the case of natural tRNA genes.

R. Percudani, unpublished observations.

Figure legends

Figure 1. Distribution of tDNA terminator length among different eukaryotes. Frequency distribution analysis of poly(dT) terminator length was conducted on the subsets of tDNAs (from S. cerevisiae, S. pombe, M. musculus and H. sapiens genomes) possessing a single Pol III termination signal starting within the first 40 bp downstream of the mature tRNA coding region. Such subsets comprised 148, 76, 214 and 250 tDNAs in the case of S. cerevisiae, S. pombe, H. sapiens and M. musculus, respectively. Upper panel: T-run length distribution in the yeasts S. cerevisiae and S. pombe. Lower panel: T-run length distribution in H. sapiens and M. musculus.

Figure 2. In vitro transcription of yeast tRNA genes displaying T₅ as the first termination signal. (A) Nucleotide sequences of the terminator regions of 8 yeast tRNA genes displaying T₅ as the first encountered termination signal. The sequences are aligned on the T₅ terminator (underlined). The sequence corresponding to the mature tRNA is in bold and italic. For tDNA #1, the sequence of a 44-bp tract (location indicated) has been omitted. (B) In vitro transcription of the templates in panel A. Lanes 1-8 refer to the templates identified by the same number in panel A. All the tDNAs except #2 possess a downstream back-up terminator that allows to monitor polymerases reading through T₅; template #2 was linearized 121 bp downstream of T₅ in order to reveal readthrough events as run-off transcripts. Left panel: the transcription reactions were carried out in the presence of 25 µM UTP; right panel: the transcription reactions were carried out in the presence of 250 µM UTP. The percentage of tDNA transcripts terminated at T₅ is reported below each lane.

Figure 3. Termination properties of 3’-flank variants of L(CAA)LR2 and N(GTT)CR tDNAs. (A) Nucleotide sequence of the terminator-surrounding regions of L(CAA)LR2 and N(GTT)CR tDNAs and their 3’-flank variants. The T₅ terminator is in bold and underlined. When present, the back-up terminator is in bold character. The L₃Δ’ and N₃A’ constructs were both linearized 16 bp downstream of T₅ in order to reveal read through events by the presence of run-off transcripts. (B) In vitro transcription assay (conducted in the presence of 250 µM UTP) of the templates reported in panel A. The arrows on the left and on the right indicate the migration positions of transcripts terminated at the T₅ element of L(CAA)LR2 and N(GTT)CR, respectively.

Figure 4. Properties of the L(CAA)LR2 T₅ terminator fused with different 3’-flanking sequences. (A) Nucleotide sequence of the L(CAA)LR2 variants resulting from mutagenesis of the region downstream of T₅. Only the sequence 3’ to the T₅ terminator (in bold and underlined) is reported. The back-up terminator is in bold character. Templates 1-3 are the same as in Figure 3. (B) The lower part shows the results of in vitro transcription assays of the templates listed in panel A (identified by the numbers above the lanes). The migration positions of the different tDNA transcription products, terminated at the first or the second T-run, are indicated on the right, together with the position of a 415-nt long DNA used as a recovery marker (RM). Reported in the upper part is a bar plot deriving from quantification of three independent experiments. Pol III transcriptional readthrough at the first terminator was calculated on the basis of the ratio between tDNA transcripts terminated at the first and second T-run. In each experiment, the transcriptional readthrough observed with the L₃’N template (template #2) was set to 100 and the effect of the mutations was evaluated as relative T₅ readthrough with respect to L₃’N and reported on the y-axis, with error bars indicating the standard error.

Figure 5. Transcriptional readthrough at a T₅ terminator is influenced by the concentration of the next incoming nucleotide. In vitro transcription assays of the L(CAA)LR2, L₃’N and L₃’GT4 templates
(templates #1, #2 and #12 of Figure 4, respectively) were carried out in the presence of either standard concentrations of NTPs (500 µM ATP, CTP, GTP; 250 µM UTP; lanes 1-3), low CTP concentration (50 µM; lanes 4-6) or low GTP concentration (50 µM, lanes 7-9). The migration positions of the transcripts terminated at the first or second T-run are indicated on the right. The percentage of tDNA transcripts terminated at T₅ is reported below each lane.

**Figure 6.** Upstream sequence effect on T₅ terminator strength *in vitro* and *in vivo*. (A) *In vitro* transcription of the SCR1Δ₁₇ and SCR1Δ₁₃'L templates was carried out in the presence of 250 µM UTP (lanes 1,2), 25 µM UTP (lanes 3,4) or 2.5 µM UTP (lanes 5,6). The migration positions of transcripts terminated at the first or second T-run of the SCR1-derived templates are indicated on the right. (B) *In vivo* expression profiles of the SCR1Δ₁₇ and SCR1Δ₁₃'L templates. Total RNA was extracted from yeast cells (YPH500 strain) transformed with YEp352 carrying the template indicated above the lanes. Fractionated RNA samples (10 µg each) were probed with a radiolabeled oligonucleotide annealing within the first 90 bp of the SCR1 RNA product. The migration positions of the transcripts derived from the chromosome-encoded SCR1 gene and from the two plasmid-borne SCR1-derived templates (1st T, 2nd T) are indicated on the right.

**Figure 7.** Transcriptional readthrough of the T₅ terminator is an intrinsic property of Pol III. (A) *In vitro* transcription of L(CAA)LR2, L₁₃'N and SCR1Δ₁₃'L derivatives fused to the 5'-flanking region of SNR6 was carried out at 250 µM UTP either in the presence (lanes 1-3) or in the absence (lanes 4-6) of TFIIIC. The percentage of transcripts terminated at the T₅ element is reported below each lane. All the shown lanes were part of the same transcription gel. However, lanes 4-6 derive from a ~10-fold darker exposure than lanes 1-3, to compensate for the lower transcription output obtained in the absence of TFIIIC. The migration positions of the transcripts terminated at the first or second T-run of either the SCR1-derived template (1st T(SCR), 2nd T(SCR)) or the L(CAA)LR2-derived templates (1st T(LEU), 2nd T(LEU)) are indicated on the right. (B) 3'-overhanged restriction fragments containing the L(CAA)LR2, L₁₃'N and SCR1Δ₁₃'L templates were transcribed *in vitro* in the presence of purified Pol III and either 25 µM (lanes 1-3) or 250 µM (lanes 4-6) UTP. The percentage of transcripts terminated at the T₅ element is reported below each lane. The migration positions of the transcripts terminated at the first or second T-run of either the SCR1-derived template (1st T(SCR), 2nd T(SCR)) or the L(CAA)LR2-derived templates (1st T(LEU), 2nd T(LEU)) are indicated on the right, together with the migration position of run-off transcripts (RO).

**Figure 8.** T₆ readthrough within a natural Pol III transcription unit. (A) *In vitro* transcription of the SNR52 gene and of the SNR52_mutT variant (in which the internal T₆ element is disrupted). The reactions in lanes 1-2 were carried out in the presence of 25 µM UTP. The reactions in lanes 3-4 were carried out in the presence of 250 µM UTP. The migration positions of the full-length primary transcript and of the T₆-terminated transcript are indicated on the left. (B) *In vitro* transcription of the 5'U₆-SNR52 template was carried out either in the presence (lanes 1, 2) or in the absence (lanes 3, 4) of TFIIIC. The reactions mixtures contained either 25 µM UTP (lanes 1, 3) or 250 µM UTP (lanes 2, 4).
Figure 1

**Upper panel:**
- **S. cerevisiae** (solid line with dots)
- **S. pombe** (dashed line with dots)

**Lower panel:**
- **H. sapiens** (solid line with dots)
- **M. musculus** (dashed line with dots)
Figure 2

A

1. tRNA_i_L (CAA) LR2
   CAACCAATTATTTTGCCCTTCCGAAAAATTTTTGA [44bp] ATCTTTTTTCTCT...
2. tRNA_P (AGG) CR
   CCCACATTGTGTACAAATGTGTGTCGCTACTGGCAGAAATGGTACGG...
3. tRNA_N (GTT) CR
   GGTGCTAAATTTTCTTTTCTTTTTTTTTTTTTATCTCCGACGGAGAAAA...
4. tRNA_S (AGA) EL
   TTGTGCTTAATTTTCTCTTTTTTTCAATTTCCCTTGTGTCGATCGAGGATGAG...
5. tRNA_M (CAT) E
   CGGCGCTAAATTTTCTTTTTTTGCCCCAACAAATACACAGATCGCAAA...
6. tRNA_N (GTT) KL
   GGTGCTATTTTTAACTTTTTTACACTGAGAAACAAATCAATCTAATA...
7. tRNA_N (GTT) OL
   GGGTCTGTAATTTTTGAGAACAAATTTCTTCTCAGACAAC...
8. tRNA_V (AAC) MR2
   GCGAAATCTAAATTTATCTACGCCGTAGCGGAAGTTTTTTTACGCCGCTT...

B

[UTP]=25µM [UTP]=250µM

% termination at T₅

1 2 3 4 5 6 7 8
99 100 65 63 95 99 96 100
98 100 17 24 87 98 94 100
### A

|   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|
| 1 | L (CAA) LR2 | …CAACCATTAA_TTTTTTGCCTTCCGAAAAATTTTGAAG […] ATCTTTTTTTT… |
| 2 | L_3’N | …CAACCATTAA_TTTTTTCTTTTCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT |
| 3 | L_Δ3’ | …CAACCATTAA_TTTTTTGGGGCGCGCCGGATC……………………………… |
| 4 | N (GTT) CR | …GGTCGTTAA_TTTTTTCTTTTCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT |
| 5 | N_3’L | …GGTCGTTAA_TTTTTTGCCTTCCGAAAAATTTTGAAGTTTTTTTTTTTTTTTTTTTTTTT |
| 6 | N_Δ3’ | …GGTCGTTAA_TTTTTTGGGGCGCGCCGGATC……………………………… |

### B

![Image of gel electrophoresis result](image-url)
Figure 4

A

1  TTTTTGCCCTTCCGAAAAATTTTA[44bp]ATC TTTTTTTT
2  TTTTTCTTTTTCTTTTTTTTTTTTTTTTT
3  TTTTTGGGGCGCCGAGCTTAAATTAGCTAGA...
4  TTTTTGCCCTTTTTTTTTTTTTTTTTTTTT
5  TTTTTCTTTTTGACTCGAATTTTTTTTTTTTT
6  TTTTTCAAAACTTTTTTTTTTTTTTTTTTT
7  TTTTTCTATATCTTTTTTTTTTTTTTTTTTT
8  TTTTTCTATATCTTTTTTTTTTTTTTTTTTT
9  TTTTTCTATATCTTTTTTTTTTTTTTTTTTT
10 TTTTTACACTTTTTCTTTTTTTTTTTTTTTTT
11 TTTTTATTTTCTTTTTTTTTTTTTTTTTTT
12 TTTTTGTCTTTTTTTTTTTTTTTTTTTTT
13 TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
14 TTTTTCTTTTTTTTTTTTTTTTTTTTTTTTT
15 TTTTTCTTTTTTTTTTTTTTTTTTTTTTTTT
16 TTTTTCTTTTTTTTTTTTTTTTTTTTTTTTT

B

![Bar chart showing Relative T5 readthrough](chart.png)

![Image of gel electrophoresis](gel.png)

- RM
- 1st T
- 2nd T
Figure 5

L(CAA)LR\textsubscript{2} L(CAA)LR\textsubscript{2} L(CAA)LR\textsubscript{2} L\_\textsubscript{3’N} L\_\textsubscript{3’N} L\_\textsubscript{3’N} L\_\textsubscript{3’GT\textsubscript{4}} L\_\textsubscript{3’GT\textsubscript{4}} L\_\textsubscript{3’GT\textsubscript{4}}

% termination at T\textsubscript{5} 96 55 78 96 86 79 100 50 83

1st T 2nd T

standard low CTP low GTP

L\_\textsubscript{AAL}R\textsubscript{2} L\_\textsubscript{3’N} L\_\textsubscript{3’GT\textsubscript{4}} L\_\textsubscript{AAL}R\textsubscript{2} L\_\textsubscript{3’N} L\_\textsubscript{3’GT\textsubscript{4}} L\_\textsubscript{AAL}R\textsubscript{2} L\_\textsubscript{3’N} L\_\textsubscript{3’GT\textsubscript{4}}
Figure 6

A

[UTP](µM) 250 25 2.5

SCR1 Δ T7
SCR1 Δ 3L
SCR1 Δ T7
SCR1 Δ 3L
SCR1 Δ T7
SCR1 Δ 3L

% termination at T5/T7 100 59 100 89 100 97

1st T
2nd T

B

SCR1

2nd T
1st T
Figure 8

A

SNR52_wt
SNR52_mutT

SNR52_wt
SNR52_mutT

Full length

T6

[UTP] (µM) 1 2 3 4

25 250 25 250

B

TFIIIC

5'U6_SNR52

[UTP] (µM) 1 2 3 4

25 250 25 250