Supporting Materials and Methods

DNA Templates. The *Saccharomyces cerevisiae* tDNA G(TCC)NL (compare http://mips.gsf.de/proj/yeast/rna/trna.html for tDNA nomenclature) was PCR-amplified from yeast genomic DNA (strain S288C) by using the high-fidelity Deep Vent DNA polymerase (New England Biolabs) and the following oligonucleotide primers: G(TCC)NL_fw (5'-CCCTTTTAAGTAATCGCTGTG) and G(TCC)NL_rev (5'GATTAGGGCTTTATGACTTCCC). The 238-bp amplified fragment was inserted, in both orientations, into the pGEM–T Easy vector (Promega) to produce pGEM-G(TCC)NL_plus (in which the tDNA 3' end points toward the *Nae*I site of the vector) and pGEMG(TCC)NL_minus (in which the tDNA 5'end points toward the *Nae*I site). The *S. cerevisiae SNR6* gene was then PCR-amplified from the pB6 plasmid (1) with the oligonucleotide primers U6_fw (5'-GTAGCATGAATACTAAATCG) and U6_ΔB_rev (5'CAAGTCACGATACTTCACTCG). The 284-bp-amplified fragment, containing the U6 RNA coding sequence plus 140 bp of 5'-flanking sequence and 15 bp of 3'-flanking sequence (U6ΔB, lacking the extragenic B-block) was inserted, in both orientations, into the *Nae*I restriction site of pGEM-G(TCC)NL_plus (located at 362 bp from the *Eco*RI site of the vector) to give the U6/tDNA constructs of Fig. 2 *B* and *C*. The same fragment was also inserted into the *Nae*I site of pGEM-G(TCC)NL_minus, to give the U6/tDNA construct of Fig. 2*D*.

The U6ΔB_300 and U6ΔB_520 templates were derived from plasmid-borne U6ΔB by inserting, into the *Eco*NI restriction site present in the U6 RNA coding region, pNEB193 vector-derived DNA fragments of 186 and 406 bp, respectively. The same DNA fragments were inserted into the *Eco*NI site of *SNR6* contained in the pB6 plasmid to generate *SNR6*_300 and *SNR6*_520. The U6-SCR1 and U6-SCR1mini variants of *S. cerevisiae SCR1* were created by mutagenic PCR using the C4T *SCR1* point mutant (2) as a template and the forward primer 5'-TTTCGGCTACTATAAATAAATGTTTTTTTCGCAACTAGCTAGGTTGTAATGGCTTTC.

Purification of rBdp1. A protein extract from insect cells was prepared in buffer I (50 mM Tris•HCl, pH 7.5/100 mM NaCl/20% glycerol/5 mM 2-mercaptoethanol/1× protease inhibitor mixture) as described (3) and then adjusted to 350 mM NaCl. The extract was subjected to chromatography on a 5-ml heparin Hyper D column (Biosepra S. A., Process Division of Ciphergen, Cergy-Saint-Christophe, France) equilibrated in buffer I. After extensive washing, bound proteins were eluted with a 50-ml linear gradient of NaCl from 0.35 to 1 M NaCl. Fractions containing His-Bdp1 were pooled and further purified by a metal chelate affinity column (POROS MC 20, Applied Biosystems) charged with Ni^{2+}. The resin was previously equilibrated with buffer I containing 500 mM NaCl, which corresponds to the ionic strength of the pooled fractions. After an 8-ml wash with buffer I containing 50 mM imidazole, rBdp1 was eluted with buffer I containing 300 mM imidazole. The full-length Bdp1 polypeptide constituted >60% of the total protein in the final rBdp1 preparation, as judged by SDS/PAGE analysis followed by Coomassie blue staining.

Purification of *S. cerevisiae* Pol III. Pol III was purified from the supernatant fraction generated during the preparation of yeast nuclear extracts, after hypotonic lysis of the spheroplasts (4). Proteins (≈1 g) of the supernatant fraction derived from ≈150 g (wet weight) of yeast cells were adsorbed in batch to 400 ml of Bio-Rex 70 (Bio-Rad) equilibrated in TEG1 buffer (25 mM Tris•HCl, pH 7.8/10% glycerol/0.1 mM EDTA/5 mM 2-mercaptoethanol) containing 50 mM $(NH_4)_2SO_4$ and protease inhibitors (1 μM leupeptin/1 μM pepstatin/0.5 mM PMSF/0.5 mM benzamidine). After extensive washing with TEG1 buffer containing 0.1 M $(NH_4)_2SO_4$, bound Pol III was eluted with the same buffer containing 0.6 M $(NH_4)_2SO_4$. The active fraction (300 mg total protein) was diluted to 0.23 M $(NH_4)_2SO_4$ and applied to a 50-ml Heaprin Hyper D column (Biosepra S. A.) equilibrated in TEG1 buffer with 0.23 M $(NH_4)_2SO_4$. After extensive washing with the same buffer, Pol III activity was eluted with a linear gradient from 0.23 to 0.83 M $(NH_4)_2SO_4$ in TEG1 buffer. Active fractions (7 mg of total protein) were pooled, dialyzed to lower salt concentration, and applied to a 9-ml DEAE-Sephadex A-25 column (Amersham Biosciences, Piscataway, NJ) equilibrated in TEG2 buffer (25 mM Tris•HCl, pH 7.8/20% glycerol/0.25 mM EDTA/5 mM 2-mercaptoethanol/protease inhibitors) containing 0.2 M $(NH_4)_2SO_4$. After extensive washing with the same buffer, Pol III was eluted with a linear gradient from 0.2 to 0.6 M $(NH_4)_2SO_4$ in TEG2 buffer. Active fractions, containing homogeneous yeast Pol III as judged by SDS/PAGE, were concentrated to 0.23 mg/ml by heparin-ultrogel chromatography.

tDNA^{Gly} Occupancy by Active Transcription Complexes in the in Cis Competition Experiment (**Fig. 6**). To evaluate the in cis character of the competition experiments shown in Fig. 2, parallel controls of tDNA^{Gly} occupancy by functional PICs were carried out, as follows. The plasmid containing the U6 and tRNA^{Gly} genes was first incubated with TFIIIB (20 min, 20°C), then TFIIIC and fresh TFIIIB were added for additional 20 min. The amounts of TFIIIC and TFIIIB components, at each addition, were as indicated in *Materials and Methods*. The number of PICs assembled on the tDNA at this point were considered as representative of the number of PICs assembled on tDNA during the competition protocol in Fig. 2. A vast excess of Pol III was then added, together with a tDNAGly-specific subset of NTPs (A, G, and U: with these NTPs, no RNA can form on the U6 gene), for 10 min at 20°C. CTP was finally added together with heparin. The products of this single-round transcription reaction, conducted in triplicate, consisted of pre-tRNAGly for the 99%, and were taken to represent the number of PICs assembled on tDNAGly. These transcripts were quantified by PhosphorImaging, and a ^{32}P-labeled DNA fragment of known specific radioactivity, loaded on the same gel, was quantified in parallel. From the comparison of the tRNA^{Gly} signal with the marker signal, we deduced the ^{32}P-derived dpm contained in the tRNA signal and, consequently, the corresponding amount of transcripts. This amount resulted to be 5.2 and 4.9 fmol in two independent determinations, respectively. Because 20 fmol of plasmid DNA are present in the reaction mixture, the calculated tDNAGly occupancy was 25%. The real occupancy may actually be higher than 25%, because it has been documented that there can be a fraction of Pol III molecules that are inactive for productive initiation but still bind to the PIC (5). Therefore, in the competition experiment of Fig. 2, at least 25% of the U6 PICs have a tDNA PIC in cis. The competition experiments in Fig. 2 *B–D* show that the U6 to tDNA transcription ratio is already 7 after 30 s, i.e., after two transcription cycles: we take this

value as an indication that a low amount of Pol III-containing complexes can assemble on the tDNA during step 3 of the competition scheme in Fig. 2*A*: this amount corresponds to one-seventh of the complexes assembled on the U6 gene. In a purely stochastic model for facilitated reinitiation, it is assumed that only the Pol III molecules originally present on a U6 gene having a competitor tDNA PIC in cis will redistribute on the two adjacent PICs after the first cycle, in function of the relative affinity of Pol III for the two PICs, which is estimated to be two times higher for the tDNA PIC than for the U6 PIC (as deduced from the 2-fold preference for the tDNA in the control experiments in Fig. 2 *B*–*D*, lanes 1-4). We thus simulated the outcomes of stochastic reinitiation by assuming that, at each cycle (i.e., approximately every 15 s): (*i*) all of the U6 complexes without any competitor in cis reinitiate on the same U6 gene; (*ii*) of the U6 complexes having a tDNA complex in cis, one-third reinitiate on the same U6, two-thirds on the tDNA (stochastic redistribution, but taking into account the unequal Pol III recruiting abilities of U6 and tDNA PICs, revealed by the control experiments in Fig. 2 *B*–*D*); (*iii*) the preassembled tDNA complexes reinitiate on the same tDNA, with the exception of those among them that happen to have a U6 complex in cis: we assume that these are ≈25%, and that at each cycle two-thirds of them reinitiate on the same tDNA, one-third on the U6 complex *in cis*. On the basis of the above scheme, we generated the plots in Fig. 6, that report the expected U6/tDNA transcript ratios in the presence of different degrees of tDNA occupancy, as compared with the values experimentally observed in Fig. 2*D*. There is a clear discrepancy between observed and theoretically predicted U6/tDNA transcript ratios. In particular, with a 25% occupancy of the tDNA by functional PICs, the U6/tDNA transcript ratio predicted by the above stochastic model is 3.42 after 4 cycles, and 2.94 after 20 cycles. The predicted ratios tend to be even lower if we consider that a 25% PIC co-occupancy is a worst-case scenario (5). The fact that a constantly 7-fold higher U6 transcription is observed during the first 20 cycles thus argues in favor of the existence of a specific mechanism of Pol III recapture by the same gene for multiple cycles.

1. Burnol, A. F., Margottin, F., Schultz, P., Marsolier, M. C., Oudet, P. & Sentenac, A. (1993) *J. Mol. Biol.* **233,** 644-658.

2. Dieci, G., Giuliodori, S., Catellani, M., Percudani, R. & Ottonello, S. (2002) *J. Biol. Chem.* **277,** 6903-6914.

3. Dumay-Odelot, H., Acker, J., Arrebola, R., Sentenac, A. & Marck, C. (2002) *Mol. Cell. Biol.* **22,** 298-308.

4. Dieci, G., Duimio, L., Coda-Zabetta, F., Sprague, K. U. & Ottonello, S. (1993) *J. Biol. Chem.* **268,** 11199-11207.

5. Bartholomew, B., Braun, B. R., Kassavetis, G. A. & Geiduschek, E. P. (1994) *J. Biol. Chem.* **269,** 18090-18095.