Supporting Information

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SI Text

Oligonucleotides, Plasmids, and Yeast Strains. The sequences of the oligonucleotides used in this study are available upon request. The coding sequence of *SUB1* was subcloned in the pScodon1 vector (Eurogentec) to give the pScodon-*SUB1* plasmid. The DNA encoding 6His-Sub1 was subcloned in the yeast expression vector pCM189 (1) to give the pCM-*SUB1* plasmid used in this study. The DNA encoding 6His-Sub1 was subcloned in a pFastbac1 vector (invitrogen) to give the pFastbac-*SUB1* plasmid. The *SUB1* gene was inactivated or epitope-tagged in different strains as described (2), and modifications of *SUB1* were checked both by PCR analysis with at least 2 sets of primers and Western blot analysis.

Expression and Purification of Recombinant Sub1. The pFastbac1-Sub1 plasmid was used for bacmid production according to the manufacturer's protocol. Expression and protein extraction were performed as described (6). High 5 extracts expressing 6His-Sub1 were subjected to chromatography on POROS MC 20 (Applied Biosystems) charged with nickel equilibrated in 20 mM Hepes, pH 7.5, 100 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol and protease inhibitor mixture (Complete, Roche Applied Science). After washing, proteins were eluted with the same buffer complemented with 300 mM imidazole (pH adjusted to 8). Fractions containing 6His-Sub1 were pooled and further purified on POROS 20 HS (Applied Biosystems) equilibrated in 20 mM Hepes, pH 7.5, 200 mM NaCl, 10% glycerol and 5 mM β-mercaptoethanol. Proteins were eluted with a 20-column volume linear gradient of NaCl from 0.2 to 1 M. Fractions where rSub1 represents >90% of the total protein content, as judged by SDS/PAGE analysis and Coomassie blue staining, were pooled. Highly purified rSub1 was injected into rabbits and the serum was collected after several immunizations. Recombinant PC4 was overexpressed and purified as described (7).

Chromatin Immunopurifications Analyzed by PCR or DNA Microarray Hybridization. ChIP, PCR, and DNA microarray hybridization were performed as described (4, 8). For ChIP on chip experiments, the immunopurified DNA and DNA from the whole-cell extracts of 3HA-Sub1 cells, fluorescently labeled, were competitively hybridized to DNA microarrays harboring 14,172 yeast ORF and intergenic regions. We checked by Western blot analysis that the absence of Sub1 did not significantly change the amounts of TFIIIC and TFIIIB in these extracts

Microrray Data Analysis. Hybridized arrays were scanned by using a GenePix 4000A scanner (Axon Instruments) and fluorescence ratio measurements were determined with the GenePix Pro 6.0 software (Axon Instruments). All array analyses were undertaken by using the Limma package (9) from the R/Bioconductor software (10). ChIP on chip spot intensities have been normalized by using the weighted median as implemented in the *normalizeWithinArrays* function of the Limma package. Normalized measures served to compute the log2-ratio for each probe. To identify enriched probes, we have used a moderated *t* test. The moderated *t test* applied here was based on an empirical Bayes analysis and was equivalent to shrinkage (or expansion) of the estimated sample variances toward a pooled estimate, resulting in a more stable inference. However, a one-sided alternative hypothesis was considered, because we expected higher intensity signal levels in the IP-enriched hybridizations than in the negative control hybridizations.

Gel Shift Assays. Protein–DNA interactions were monitored by gel shift assays as described (6) using a ^{32}P-labeled DNA fragment carrying the tRNA^{Ileu} or the *SUP4* tRNA^{Tyr} genes as probes. The amounts of nonspecific DNA plasmid competitor (pBluescript KS) used for EMSA with recombinant fractions (Fig. 3 *A*, *C*, and *D*) was correlated to the amount of Sub1 used in each lane (ratio in ng, Sub1/competitor DNA of ≈2/1), so that its non specific DNA binding activity was not detected. Note that when rTFIIIC and rTFIIIB alone or in combination were used, approximately only 1–3 ng of DNA plasmid competitor was used. When yeast TFIIIC was used (Fig. 3*B*), 100 ng of DNA plasmid competitor were added to the reaction. For supershift experiments, preassembled complexes were further incubated at 25 °C during 20 min with 600 ng of anti-Histidine (Qiagen 34660) or anti-Flag M5 (Sigma F-4042) monoclonal antibodies.

In Vitro Transcription Assays. The 17-mer assays have been performed as described (11). The 17-, 15-, and 14-mer RNA visualized in Fig. 2*B* are the most abundant transcripts generated in single round transcription assays (12, 13). For each reaction the amounts of these transcripts were quantified and summed up, using the Quantity One software (Bio-Rad), to estimate transcription initiation levels. Reinitiation assays on the *SUP4*-tDNA^{Tyr} or the tDNA^{Ileu} (TAT) genes were performed as described (14) with the amounts of proteins used for multiple round of transcription assays, excepted when indicated. Transcriptions with crude extracts (50 μg) were incubated for 60 min at 25 °C, in the presence of 40 ng of the plasmid template.

Far Western Blot. The pScodon-*SUB1* plasmid was linearized with BamHI and used in a TNT transcription/translation kit (Promega) in the presence of [^{35}S]methionine to generate a labeled Sub1. rSub1, rPC4, rTFIIIA, rTFIIIC, rTBP, rBrf1 and rBdp1 were subjected to SDS/PAGE and blotted onto nitrocellulose. Hybridization and washes were performed as described (15) except that the rSub1 probe was purified on MicroSpin G-25 columns (GE Healthcare) and that the membranes were first stained with Ponceau S (Sigma) then destained with water before their incubation with the blocking buffer. Labeled proteins were revealed by autoradiography or visualized with a Typhoon 9200 Imager (Amersham Biosciences).

Pulse Labeling. Wild type and *sub1*Δ cells transformed with pCM189 vector or pCM-*SUB1* plasmid were grown in Casamino Acids medium supplemented with adenine (20 μg/mL) to midlog phase (OD_{600} = 0.5). Total RNAs were labeled for 10 min by adding 150 μCi of (5,6 ^{3}H)-uracil (1 mCi/mL) to 10 mL of cell cultures as described (16). Three micrograms of total RNA were analyzed by gel electrophoresis under denaturating conditions (acrylamide 8%, urea 7 M). The gel was stained with ethidium bromide, processed for fluorography with Amplify (GE Healthcare) and then dried before autoradiography. Quantitation of images of fluorographs or stained gels, acquired with a scanner, was performed by using the Quantity One software (Bio-Rad). Transcription signals were normalized against RNA recovery as determined by analysis of stained gels. Note that similar results were observed when Sub1 was overexpressed from a multicopy plasmid.

Primer Extension Analysis. Total RNAs were extracted from 50 OD_{600} of cells in midlog phase. Primer extension experiments were performed as described (17) using 4 μg of RNA and the following oligonucleotides as primers:

35S rRNA: 5′-ACACGCTGTATAGACTAGGC-3′
$tRNA^{Ileu}$: 5′-TGCTTTTAAAGGCCTGT-3′

1. Gari E, Piedrafita L, Aldea M, Herrero E (1997) A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in Saccharomyces cerevisiae. *Yeast* 13:837–848.
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19. Dieci G, et al. (2009) Positive modulation of RNA polymerase III transcription by ribosomal proteins. *Biochem Biophys Res Commun* 379:489–493.
20. Ferrari R, Dieci G (2008) The transcription reinitiation properties of RNA polymerase III in the absence of transcription factors. *Cell Mol Biol Lett* 13:112–118.

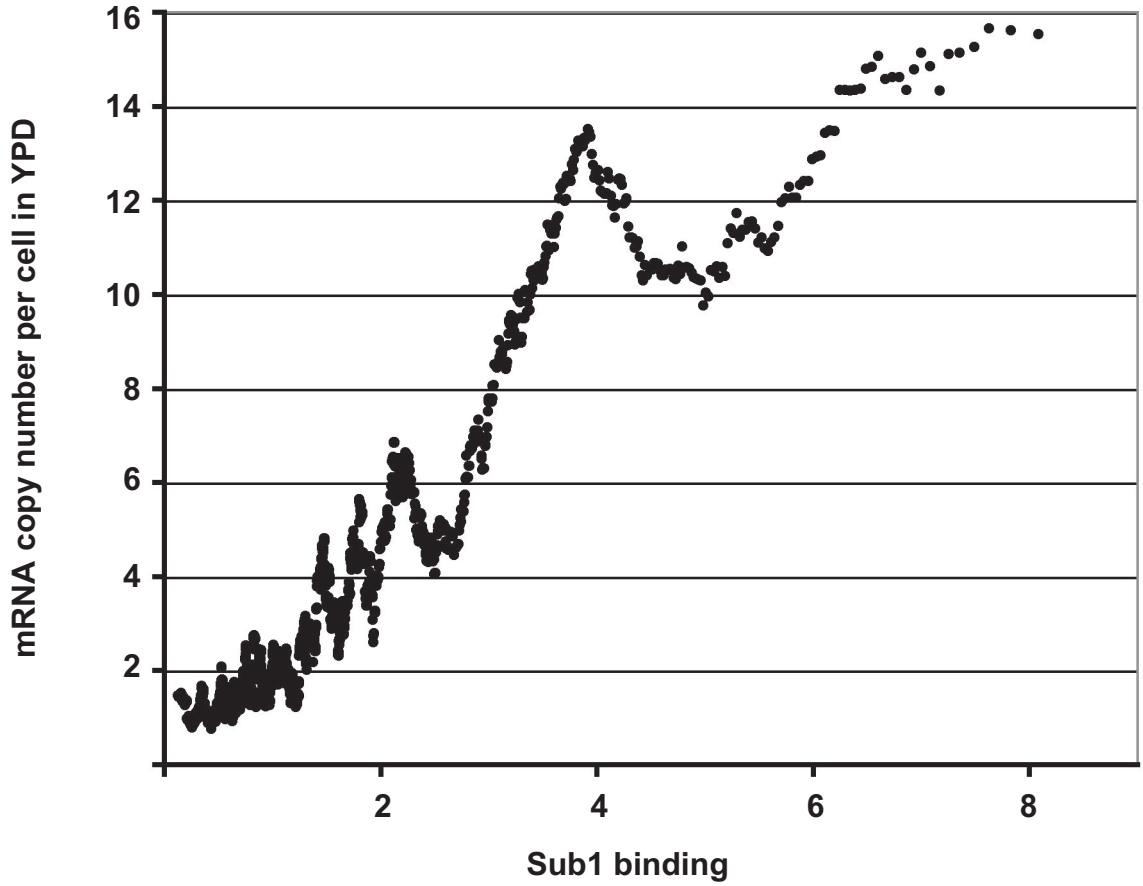

Fig. S1. Sub1–3HA binds preferentially to ORF with high transcriptional activity. The moving average (window size = 100, step size = 1) of Sub1–3HA enrichment on an ORF is plotted as a function of mRNA molecule copy number per cell (18).

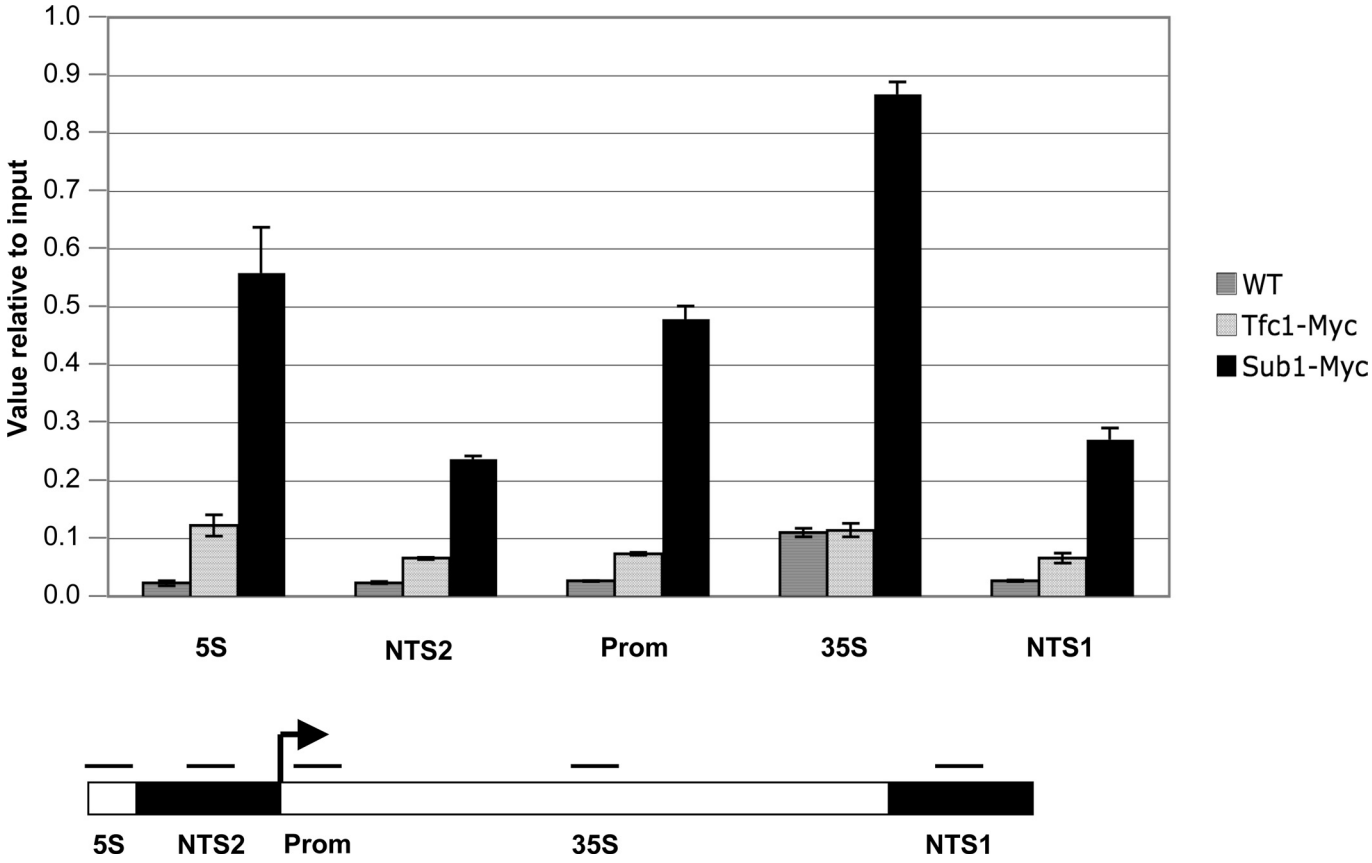

Fig. S2. Sub1 binds to the rDNA locus. Immunoprecipitations from wild type (WT), Tfc1–13myc or Sub1–13myc cross-linked chromatin were analyzed via quantitative PCR. The amounts of immunoprecipitated DNA expressed as a value relative to that of the input are shown as histograms. Sequence elements within the rDNA unit (Non-Transcribed Spacer 1 and 2, 5S DNA and 35S DNA) and the positions of the amplified DNA fragments are schematically represented. Note that although 5S DNA is transcribed by Pol III, the binding of TFIIIC to these loci could not be detected by ChIP (4).

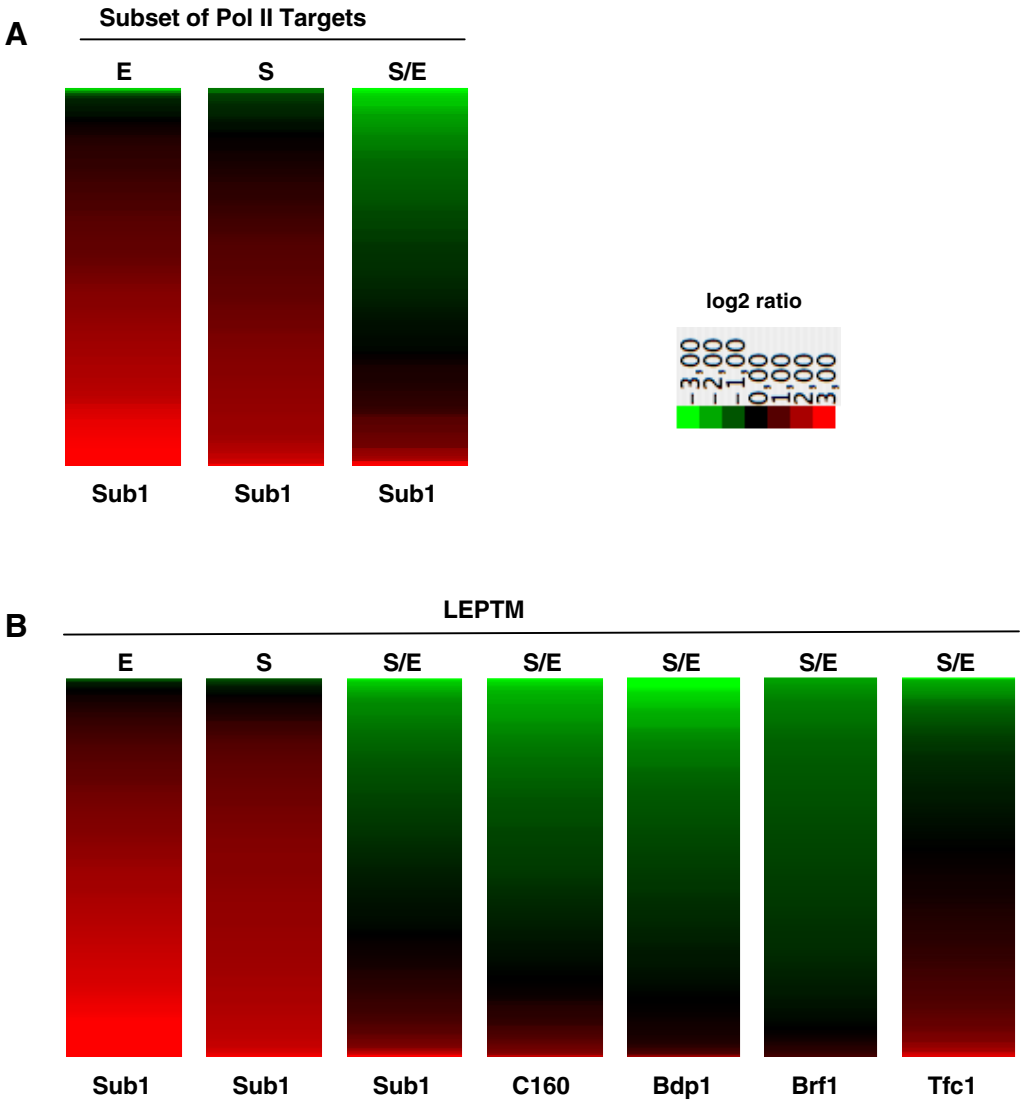

Fig. S3. Differential binding of Sub1 to a subset of Pol II targets or to the LEPTM in exponential or stationary phase. Immunopurified DNA and DNA from Sub1–3HA whole-cell extracts, prepared from cells grown to exponential or stationary phase were competitively hybridized to DNA microarrays. Sub1 occupancy in exponential (E) or stationary (S) phase or its differential binding in both growth conditions (S/E) to (*A*) a subset of Pol II targets (*ACT1*, *ADH1*, *PMA1*, *CDC19*, Histone genes and 133 RP genes) or (*B*) Pol III-transcribed genes (LEPTM) are presented according to the red-green color scale with a different gene/loci order in the *y* axis to allow the comparison with the differential binding of C160, Bdp1, Brf1 or Tfc1 to LEPTM in exponential or stationary phase published in Oficjalska-Pham et al. (2006). See also accompanying Datasets S1 and S2.

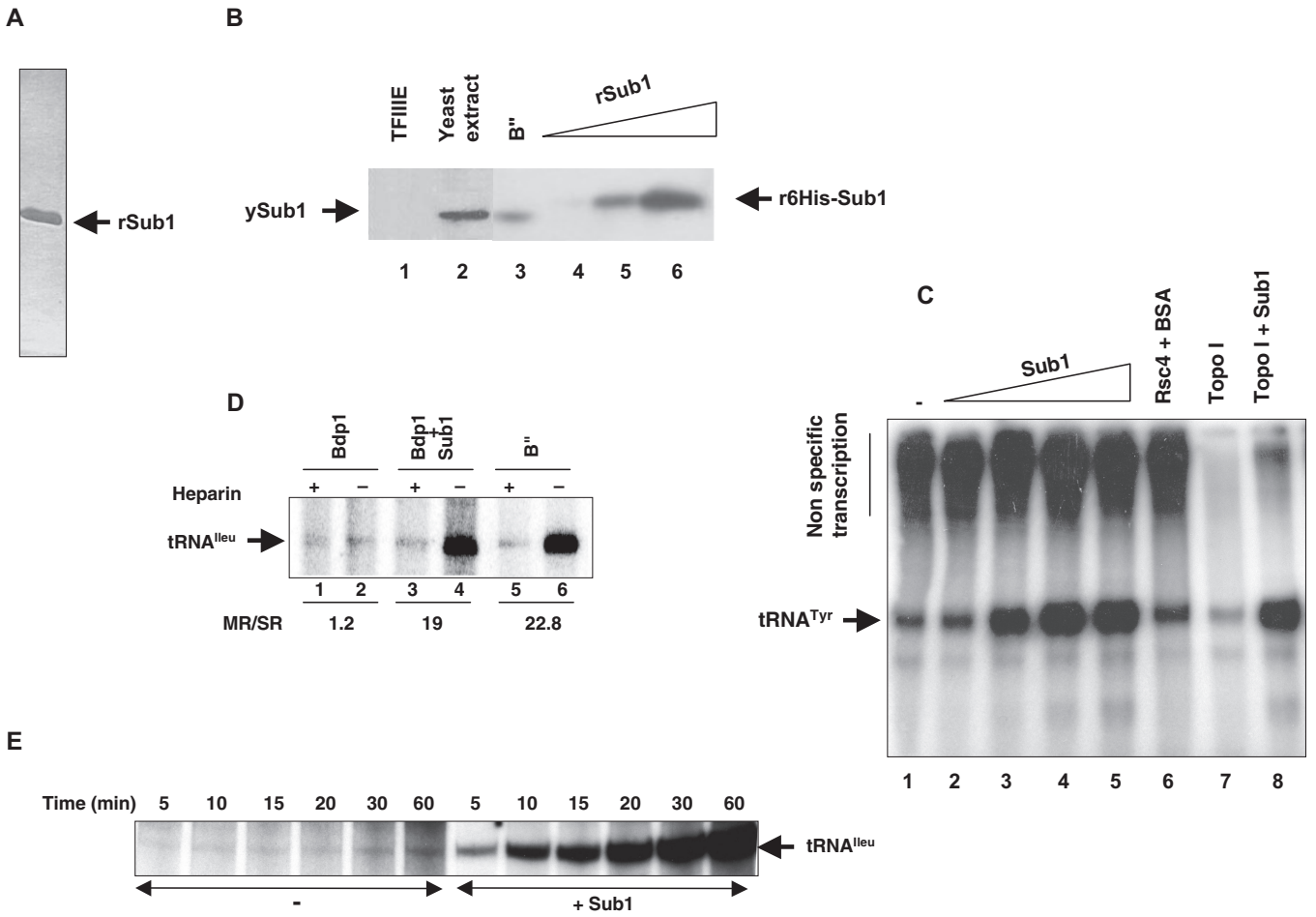

Fig. S4. Sub1 and Pol III transcription in vitro. (*A*) Purified rSub1. 2 μg of highly purified rSub1 were analyzed by 10% SDS/PAGE and Coomassie blue staining. (*B*) Sub1 is present in the B′′ fraction but not in TFIIIE. Purified TFIIIE (lane 1: 1.5 μL, ref. 19), yeast total protein extract (lane 2; 10 μg), B′′ fraction (lane 3; 2 μL), and purified rSub1 (lanes 4–6; 0.5, 3 or 10 ng) were analyzed by 10% SDS/PAGE and Western blot analysis using antibodies specific to Sub1. (*C*) Sub1 stimulates the minimal Pol III transcription system. In vitro transcription of the *SUP4*-tDNA^{Tyr} gene was carried out in the presence of rTBP, rBrf1, rBdp1, rTFIIIC, purified Pol III, varying amounts of Sub1 (lanes 2–4; 10, 30, 80 ng; lanes 5, 8; 240 ng) or 200 ng of control protein rRsc4, BSA or Topo I as indicated. The positions of the specific transcripts (tRNA^{Tyr}) and of the larger RNAs obtained after nonspecific transcription from the plasmid template are indicated. (*D*) Sub1 promotes reinitiation on tDNA^{Ileu} gene. Facilited reinitiation transcription assays were performed with the tDNA^{Ileu} template containing a TATA box under conditions described in Fig. 2C. (*E*) Time course analysis of the effect of Sub1 on in vitro transcription. rTFIIIB, rTFIIIC, Pol III, tDNA^{Ileu} template and nucleotides (in the same amounts than for Fig. S4C) were mixed together in the absence (−) or presence (+ Sub1) of 200 ng of Sub1 and incubated at 25 °C for the indicated time (min) before stopping the reaction.

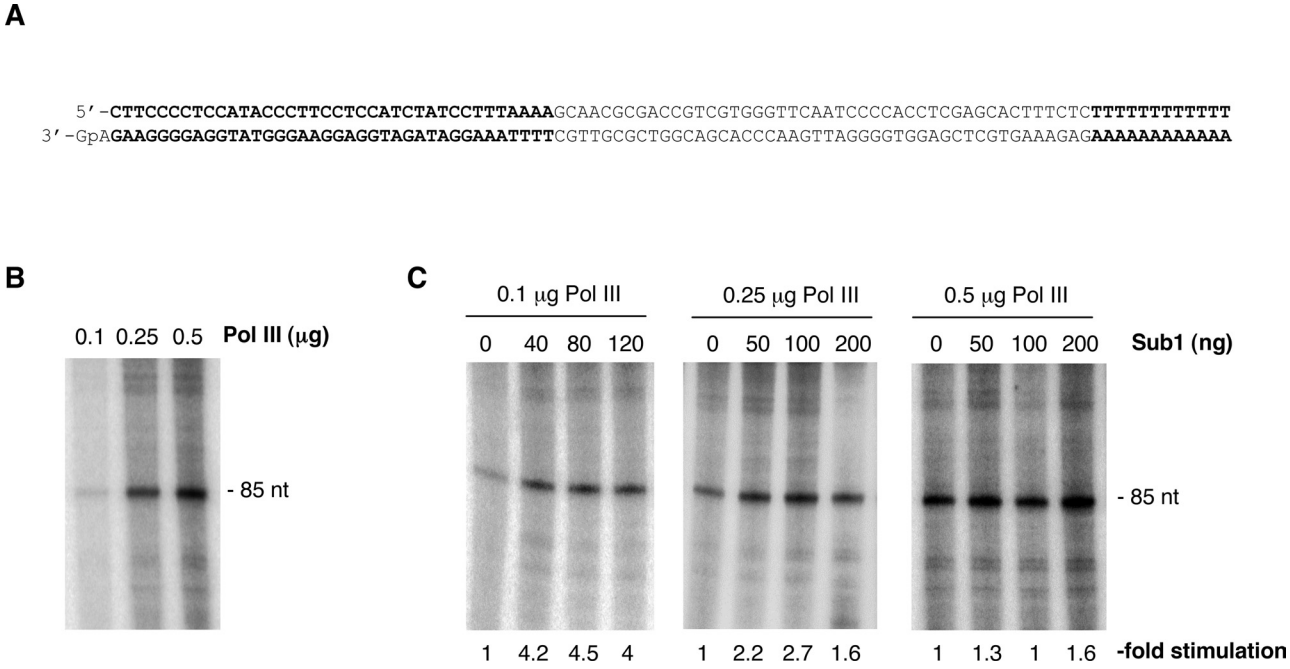

Fig. S5. Sub1 stimulates factor-independent transcription by Pol III when Pol III is in limiting amounts. (*A*) Sequence of the tDNA^{Ileu}(TAT)-derived, 3′-overhanged template used in the assay. (*B*) Transcription with increasing amounts of purified Pol III. Factor-independent transcription by Pol III was carried out as described (20) in the presence of low, intermediate or high Pol III concentrations. (*C*) Effect of Sub1 on factor-independent transcription by Pol III. Increasing amounts of Sub1 were added to the reaction mixtures as indicated in the presence of low, intermediate or high Pol III concentrations (left, middle and right subpanels, respectively). The extent of transcriptional stimulation by Sub1 is reported below each lane; it represents the average of 2 independent experiments that differed by no more than 25% of the mean.

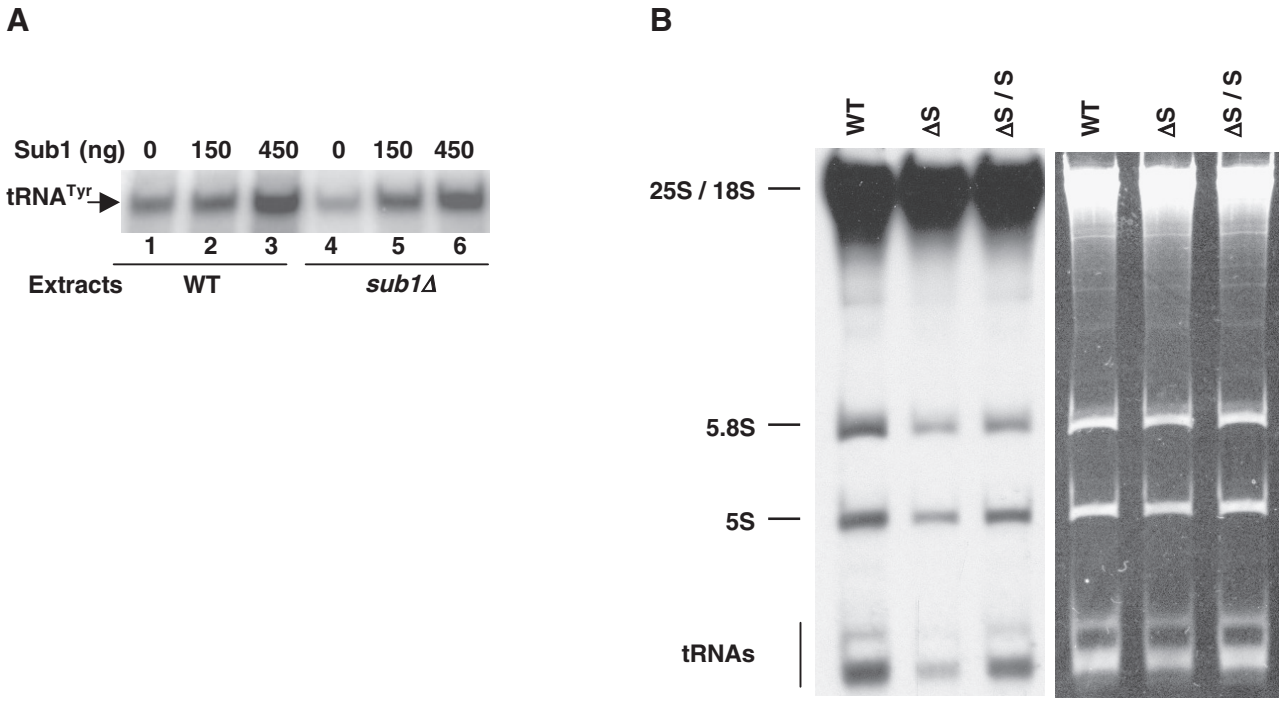

Fig. S6. Sub1 and Pol III transcription in vivo. (*A*) Crude extracts from *sub1*Δ cells are deficient in Pol III transcription. In vitro transcription of the *SUP4*-tDNA^{Tyr} gene was carried out by using extracts prepared from wild type (WT) or *sub1*Δ cells in the presence of the indicated amounts of Sub1. (*B*) The RNA synthesis by Pol III is less efficient in *sub1*Δ strain but the steady state levels of RNA are unchanged. Three micrograms of total RNA prepared from exponentially growing cultures of wild type (WT), *sub1*Δ (ΔS) or *sub1*Δ/pCM-*SUB1* cells (*sub1*Δ transformed with a centromeric plasmid harbouring *SUB1*, ΔS/S) were analyzed by electrophoresis and fluorography. (*Right*) Ethidium bromide staining of the gel. (*Left*) Fluorograph of the gel. The positions of 25S, 18S and 5.8S RNA transcribed by Pol I, and of 5S RNA and tRNAs transcribed by Pol III are indicated.

Other Supporting Information Files

Table SI (PDF)
Dataset S1 (XLS)
Dataset S2 (XLS)