Two Steps in Maf1-Dependent Repression of Transcription by RNA Polymerase III

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Running title: Maf1-dependent repression of pol III transcription
SUMMARY

In *Saccharomyces cerevisiae*, Maf1 is essential for mediating the repression of transcription by RNA polymerase (pol) III in response to diverse cellular conditions. These conditions activate distinct signaling pathways that converge at or above Maf1. Thus, Maf1-dependent repression is thought to involve a common set of downstream inhibitory effects on the pol III machinery. Here we provide support for this view and define two steps in Maf1-dependent transcriptional repression. We show that chlorpromazine (CPZ)-induced repression of pol III transcription is achieved by inhibiting *de novo* assembly of the initiation factor TFIIIB onto DNA as well as the recruitment of pol III to pre-assembled TFIIIB-DNA complexes. Additionally, Brf1 was identified as a target of repression in extracts of CPZ-treated cells. Maf1-Brf1 and Maf1-pol III interactions were implicated in the inhibition of TFIIIB-DNA complex assembly and polymerase recruitment by recombinant Maf1. Co-immunoprecipitation experiments confirmed these interactions in yeast extracts and demonstrated that Maf1 does not differentially sequester Brf1 or pol III under repressing conditions. The results suggest that Maf1 functions by a non-stoichiometric mechanism to repress pol III transcription.

Keywords: Maf1/Mechanism/Repression/RNA polymerase III/Transcription
INTRODUCTION

Transcription of the large ribosomal RNAs (rRNAs) by RNA polymerase (pol) I and of 5S rRNA and tRNAs by pol III is tightly co-regulated under essentially all conditions (1-3). This co-ordinate regulation is biologically important as it is conserved in all eukaryotes where transcription by pols I and III has been examined. The principal evolutionary imperatives that are thought to underlie this conserved regulation are the common function of rRNAs and tRNA in protein synthesis and the high energetic cost of their synthesis, which accounts for about 80% of nuclear gene transcription in actively growing cells (1;3). The levels of pol I and pol III transcription are critical determinants of cell growth rate and the deregulation of this transcription is a hallmark of cell transformation and tumorigenesis (4;5). In addition, for single cell eukaryotes whose biological niche exposes them to periods when nutrients are in short supply and/or harsh environmental conditions, the ability to rapidly shut off the synthesis of rRNAs and tRNA is thought to be of vital importance for achieving metabolic economy (6) and hence, is likely to impact cell survival.

In higher eukaryotes, p53, Rb and its relatives p107 and p130 play an important role in controlling pol I and pol III transcription and in coordinating the production of new protein synthetic capacity with cell proliferation (2;5). These repressors function by binding directly to components of the pol I and pol III transcription machinery and thereby prevent protein-protein interactions required for transcription. Specific components of this machinery are also substrates for phosphorylation by various kinases including ERK, casein kinase II (CK2) and cyclin-
dependent kinases, which can either activate or repress transcription depending on the stage of the cell cycle and the growth conditions (2;7-10). In lower eukaryotes such as budding yeast, there are no homologs of p53 or Rb and the signaling pathways that regulate pol I and pol III transcription have yet to be clearly defined (3;6;11). However, as in mammalian cells, phosphorylation is likely to play a key role. For example, pol III transcription in yeast requires Tpd3, a regulatory subunit of protein phosphatase 2A (12) as well as CK2, which is necessary for efficient TFIIB-DNA complex assembly (13). CK2 copurifies with the TBP and Brf1 subunits of the initiation factor TFIIB and phosphorylates TBP (most likely on S128, (13)). Yeast TFIIB (which comprises TBP, Brf1 and Bdp1 polypeptides) and in some cases pol III itself, has been suggested as a regulatory target based on its ability to rescue (at least partially) the transcription defect in extracts prepared from tpd3 and cka2 mutant strains or following rapamycin or cycloheximide treatment (12-15).

Recent experiments in yeast have identified a structurally novel and phylogenetically conserved protein, Maf1 (16), as an essential and specific mediator of pol III transcriptional repression under a wide variety of conditions (17). An absolute requirement for Maf1 in the repression of pol III transcription has been demonstrated in maf1Δ cells following interruption of the secretory pathway and in response to DNA damage, growth to stationary phase and treatments with rapamycin and the anti-fungal compound chlorpromazine (CPZ). As these conditions are known to activate at least three distinct signaling pathways, (secretory signaling, TOR kinase and DNA damage response pathways), it is evident that these pathways must converge upon Maf1 in order to affect repression (3;17). Consistent with this view, Maf1 is located at
or near the end of these pathways based on its direct but limited interaction with pol III (16). Given
the range of conditions that have been examined to date, the findings suggest that Maf1 may be
required universally for pol III transcriptional repression in yeast and that common mechanisms
will operate downstream of Maf1 to affect repression of the pol III transcription machinery.

In this work, we have examined the consequences of Maf1-dependent repression in vivo
and in vitro following CPZ treatment. We find that repression is affected at two different steps in
transcription, namely TFIIIB-DNA complex assembly and polymerase recruitment. Maf1 is
shown to interact with a small fraction of the Brf1 and pol III in control yeast extracts and these
interactions are not affected quantitatively under repressing conditions. The results exclude a
stoichiometric mechanism of action by Maf1 and suggest that Maf1 functions, directly or
indirectly, in a catalytic process to repress pol III transcription.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains** - All strains were derived from W303α (MATα leu2-3,112 trp1-1 ura3-1 can1-
100 ade2-1 his3-11,15 ssd1-1). Haemaglutinin (HA) or Myc tags were added to the 3’-ends of
the **TFC1, BRF1, BDP1, RPC53, RPC82 and MAF1** genes by PCR-based homologous
recombination using the plasmids pYM2 and pYM6, respectively (18).

**Extracts, transcription factors, antibodies and recombinant Maf1** - Control and CPZ-treated
whole cell extracts were prepared as described previously (17). Extracts were initially fractionated on
Bio-Rex70 to obtain the BRα fraction (19). Subsequent chromatography of the BRα fraction on
DEAE-Sephadex A-25 (19) yielded a flow-through fraction containing TFIIIB. The column was
then developed with linear gradient of ammonium sulfate (70-700 mM) to isolate the TFIIIC and pol III fractions (20). Pure recombinant TFIIIB subunits were prepared as described previously (21) and refs therein). TBP was kindly provided by Dr. Michael Brenowitz. Yeast Bdp1 with a histidine tag at its C-terminus was purified from the BRα fraction (19) under denaturing conditions on Ni\textsuperscript{2+}-NTA agarose resin (Qiagen Inc.) following the manufacturer’s recommendations. The protein was renatured from 8 M urea in 40 mM Hepes-KOH, pH 7.9, 500 mM KCl, 5 mM MgCl\textsubscript{2}, 10% glycerol, 1 mM DTT, and 0.1 mM EDTA and quantified by Western blotting with rBdp1 as a standard. Polyclonal antibodies to each TFIIIB subunit and Tfc4 have been described previously (22;23). The Rpc34 antibody was kindly provided by Dr. Steven Hahn.

\textit{S. pombe} Maf1 was PCR amplified from a cDNA library provided by Dr. Tomohiro Matsumoto. A 740 bp PCR product was inserted into the pCR 2.1- TOPO cloning vector (Invitrogen) and sequenced. For bacterial expression the entire open reading frame was cloned downstream of the Precision protease cleavage sequence in pGEX-6p-3 (Pharmacia) to yield GST-SpMaf1 and into Nde1/Xho1 cleaved pET-30a+ (Novagen) to yield a C-terminal hexahistidine tagged \textit{Sp}Maf1. GST-SpMaf1 was transformed into \textit{E. coli} strain BL21 Star (DE3) (Invitrogen) and protein expression was induced by adding 1 mM IPTG at 37\textdegree C for 2 hrs. GST-SpMaf1 (52 kDa) was purified under native conditions using Glutathione-Sepharose-4B beads (Pharmacia) as per the manufacturer’s recommendations. The histidine-tagged \textit{Sp}Maf1 construct was transformed into the Rosetta (DE3) pLysS strain (Novagen). Cultures grown at
37°C were induced for 16 hrs with 0.1 mM IPTG after shifting the temperature to 15°C. SpMaf1 (27 kDa) was purified under denaturing conditions in 8 M urea using Ni²⁺-NTA agarose beads (Qiagen) as per the manufacturer’s recommendations. Further purification was achieved by chromatography on Resource Q (Pharmacia) in 20 mM Tris-acetate pH 7.0, 100 mM sodium acetate, 0.1 mM EDTA, 1 mM DTT, 6M urea. Maf1 was eluted with a linear gradient of sodium acetate from 100 mM to 1 M. Pooled fractions were renatured by dialysis at 4°C against 20 mM Tris-acetate pH 7.0, 50 mM ammonium sulfate, 0.1 mM EDTA, 1 mM DTT and stored at -70°C.

Fragments of S. cerevisiae MAF1 containing conserved domain A (amino acids 1-200, no intron) or domains B+C (amino acids 201-395) and the full-length protein were expressed with a C-terminal histidine tag in pET-30a(+) and pET-21d, respectively, using the Rosetta pLysS strain noted above. ScMaf1(A) and (B+C) fragments were purified under denaturing conditions on Ni²⁺-NTA agarose and renatured into 50 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol. For the A domain fragment, the final urea concentration was maintained at 0.5 M. Full-length ScMaf1 was gel purified and used as a standard for quantifying ScMaf1 in yeast cell extracts. The concentrations of GST-SpMaf1 and ScMaf1(A) were determined by the BioRad microassay, while for SpMaf1, ScMaf1(B+C) and full-length ScMaf1, the absorbance at 280 nm was used together with the calculated molar extinction coefficients. Polyclonal antibodies were raised in rabbits against recombinant S. cerevisiae Maf1 (amino acids 201-395).
Maf1-Brf1 Interaction Assay - Recombinant His-tagged Brf1 was immobilized on Ni\textsuperscript{2+}-NTA-resin in binding buffer containing 50 mM sodium phosphate, pH 7.5, 5 mM magnesium acetate, 150 mM potassium acetate, and 10% glycerol with protease inhibitors (0.5 mM PMSF, leupeptin, pepstatin and aprotinin at a concentration of 2 µg/ml). Untagged versions of either full-length *S. pombe* or *S. cerevisiae* Maf1, cloned into pET-30a(+), were individually transcribed and translated *in vitro* (SML\textsuperscript{NT®}-coupled reticulocyte lysate system, Promega) to generate [\textsuperscript{35}S]-labeled proteins. Each pull-down reaction (100µl) contained control or Brf1 (100 pmole) resin (10 µl) and [\textsuperscript{35}S]-labeled Maf1 (10 µl) in binding buffer (see above) containing 10 mM imidazole, bovine serum albumin (5 µg/µl) and 0.1% Triton X-100. After incubation at 4 °C for 2 hr, the resins were washed in binding buffer containing 20 mM imidazole (3 x 20 min.) and then in binding buffer containing 30 mM immidazole, 400 mM NaCl and 0.2 % Triton X-100 (3 x 20 min.). Samples were boiled in Laemmli buffer, analyzed by SDS-PAGE and the labeled Maf1 proteins were detected by autoradiography.

Transcription and complex assembly assays - Standard multiple round transcription assays in whole cell extracts (60 min at 15 °C) were performed on a tRNA\textsuperscript{Leu}\textsuperscript{3} template (YEp13, 0.5 µg) as described (24). Transcription of the B box-deleted *SNR6* template (U6ΔB) was performed similarly except that the extracts were supplemented with rTBP (20 pmole). To prepare Brf1-immunodepleted extracts, whole cell extracts (400 µg) were incubated with an affinity purified
Brl1-antibody (8 µl) at 4°C for one hour. Protein A-Sepharose 4 fast flow (Amersham Biosciences, 10 µl), equilibrated with KBC100 (20 mM Hpes-KOH pH 7.9, 100 mM KCl, 2 mM DTT, 0.2 mM EDTA, 20% glycerol) was added and mixed at 4°C for four hours. The tube was centrifuged (14,000 rpm, 4°C, 15 sec) and the supernatant was used for transcription and Western blotting.

TFIIIC-independent transcription was reconstituted on the U6ΔB template (0.5 µg) with rTBP (20 pmole), rBrl1 (8 pmole), yeast Bdp1 (112 fmole, prepared from chromatin, (24) and pol III (0.36 µg, DEAE-purified). TFIIIC-dependent transcription on the tRNA^Leu^3 template (YEp13, 0.5 µg) was performed similarly except that TFIIIC (17 fmole, Mono Q fraction, (24) and rTBP (1 pmole) were used unless otherwise specified. Transcription was allowed to proceed for 60 min. at 20°C with or without a prior preincubation (45 min. at 20°C), conducted in the absence of nucleotides and polymerase, to assemble TFIIIB onto the DNA. Recombinant Maf1 fragments were added either before or after the formation of the TFIIIB-DNA complex.

Heparin-stripped TFIIIB-DNA, TFIIIB-TFIIIC-DNA and Brl1-TFIIIC-DNA complexes were assembled on a sup3-eST tRNA gene and resolved on native 4% polyacrylamide gels as described previously (17;25). Incubation of rMaf1 fragments with pre-assembled TFIIIB-TFIIIC-DNA complexes was carried out for an additional 20 min. prior to gel loading. rMaf1 proteins were dialyzed in complex assembly buffer for these reactions (25).

**Immunoprecipitation of Maf1, Brl1 and pol III** - Whole cell extracts (2 mg) prepared from
control and CPZ-treated cells expressing HA- and/or myc-tagged proteins were incubated with monoclonal antibodies (12CA5 or 9E10, 2 µg, Roche Biochemicals) in KBC100 buffer (see above) in a total volume of 400 µl at 4°C for 3-4 hr (Rpc82 and Maf1) or overnight (Brf1) followed by a 3 hr incubation with protein G-Sepharose beads (20 µl). The beads were washed with KBC100 buffer (3 x 15 min), boiled in SDS sample buffer and aliquots were subjected to immunoblotting.

**Chromatin immunoprecipitation and quantitative PCR** - ChIP analysis was carried out essentially as described by Kuras and Struhl (26). DNA samples were analyzed by quantitative PCR using primer pairs for three different tRNA genes and two negative controls (a ribosomal protein gene, *TCM1* and an intergenic region on chromosome V, Int V, see Supplementary Data).

**RESULTS**

**The activity of Brf1 in transcription is repressed in extracts of CPZ-treated cells**

In previous work we established an in vitro system to study Maf1-dependent repression of pol III transcription (17). Whole cell extracts prepared following CPZ treatment of logarithmically-growing cells were shown to have significantly reduced (>15 fold) pol III transcription compared to untreated cell extracts. The low transcription activity of these treated cell extracts could not be achieved by addition of CPZ directly to control extracts and did not result from the presence of an abundant repressor in the treated extracts (deduced from extract mixing experiments (17). However, extract supplementation with control yeast TFIIB substantially rescued the transcriptional defect while excess molar amounts of the assembly factor TFIIC or
pol III from control extracts had no effect. To further examine the effect of CPZ treatment in this system, TFIIB, TFIIC, and pol III were partially purified from the control and treated extracts to compare their individual activities in transcription. Western blotting for representative TFIIC and pol III subunits indicated that the corresponding control and treated fractions contained equivalent amounts of these factors per microgram of protein (Fig. 1A and B, Tfc4 and Rpc34), consistent with their levels in the unfractionated extracts (17). Subsequent titrations of the TFIIC or pol III fractions under conditions where these components are limiting for transcription revealed no significant differences in their activity (Fig. 1A and B). For pol III, this result was somewhat unexpected given the ability of Maf1 to be co-immunoprecipitated from crude extracts with the polymerase (16) and other results described below. We note however that Maf1 was resolved from pol III during purification and was not detectable in the pol III fraction by Western analysis (<10 ng Maf1/2.5 µg protein). For TFIIC, the similar activity of control and treated fractions is concordant with transcription experiments performed in TBP-supplemented crude extracts using a B box-deleted SNR6 template (U6ΔB). Under these conditions, U6ΔB transcription is TFIIC-independent (27), yet the large differential in transcription between the control and treated extracts was retained². On the basis of these findings, a direct role for TFIIC in the repression of pol III transcription by CPZ seems unlikely.

In contrast to the results described above for TFIIC and pol III, the TFIIB fractions revealed differences in both the recovery and the activity of this initiation factor. In particular, the amount of Brf1 in the CPZ-treated fraction was six-fold lower (per microgram of protein)
than in the control fraction (Fig. 1C, lower panel), despite the fact that equivalent amounts of Brf1 were present in the starting extracts (17). Differential recovery of Brf1 upon purification has previously been correlated with the activity of the starting extracts (22;24). In these cases, the higher transcription activity of extracts derived from PCF1-1 and PCF1-2 strains, which contain dominant mutations in the second largest subunit of TFIIIC, resulted in more efficient recovery of Brf1 in purified TFIIIB fractions relative to wild-type. Although the basis for this effect is not known, the same correlation is found in the current study with control and repressed whole cell extracts and their corresponding TFIIIB fractions. The different amounts of Brf1 in the purified fractions relative to other TFIIIB subunits (e.g. TBP, whose concentration in the treated fraction was only 30% lower than the control) may complicate the interpretation of transcription experiments where this fraction is limiting. Therefore, we first determined whether the activity of one or more of the TFIIIB subunits was compromised in the CPZ-treated whole cell extract by performing supplementation experiments.

Control and CPZ-treated whole cell extracts contain equal amounts of each TFIIIB subunit (17)\(^2\). Supplementation of a control extract with excess molar amounts (e3-fold) of rTBP, rBrf1 or affinity purified yeast Bdp1 resulted in negligible to small effects on transcription, which increased up to 2.6 fold when Brf1 and Bdp1 or all three TFIIIB components were added together (Fig. 2A). In contrast, the CPZ-treated extract, which was 15 fold less active than the control, produced a large (~7 fold) increase in transcription when supplemented with Brf1 alone. Further increases in transcription were observed when TBP was added together with Brf1 (~11
fold) or when all the three TFIIB subunits were added (~13 fold). By comparing the transcription activity between extracts supplemented with the same factors, it is apparent that addition of exogenous Brf1 substantially reduces (from 15 fold to 2.5 fold), but does not completely eliminate the differential between the CPZ-treated and control extracts (Fig. 2A and B). This suggests that changes in the activity of Brf1 underlie, at least in part, the transcriptional differences between the extracts. Conversely, differences in the activity of TBP and Bdp1 do not appear to contribute significantly to the transcriptional differential between the extracts.

Comparable results were obtained with extracts immunodepleted of Brf1 (Fig. 2C). A Brf1 polyclonal antibody was used to deplete about 90% of the Brf1 from control and CPZ-treated extracts. Mock-depleted extracts showed no significant reduction in Brf1 and the level of TBP and Bdp1 was not reduced in the Brf1-depleted extracts (data not shown), consistent with the ease of dissociation of these components from one another in the absence of DNA (28). Like the starting whole cell extracts (Fig. 2A and 2B), the transcription differential in the mock-depleted control and CPZ-treated extracts was reduced from 12 fold (Fig. 2C, lanes 1 and 6) to 2.8-fold (Fig. 2C, lanes 2 and 7) after supplementation with rBrf1. Transcription in the Brf1-depleted extracts was undetectable (Fig. 2C, lanes 3 and 8). However, supplementation of both depleted extracts with Brf1 rescued transcription and reduced the differential between them to less than 4-fold (Fig. 2C, lanes 4 and 9). Further supplementation with TBP produced only a small increase in transcription (Fig. 2C, lanes 5 and 10), indicating that TBP is not limiting to any significant degree in the depleted extracts.
The finding that TBP and Bdp1 do not contribute significantly to the transcriptional difference between the extracts (Fig. 2A and 2B) allowed a comparison of Brf1 activity in the partially purified control and CPZ-treated TFIIIB fractions. Titrations of the TFIIIB fractions were performed in the presence of control yeast TFIIIC and pol III fractions with normalized excess concentrations of TBP and Bdp1 (see Fig. 1C for a representative experiment). A plot of the resulting transcription expressed as a function of the amount of Brf1 in the assay revealed a 3-fold difference in the apparent specific activity of Brf1 (Fig. 1D). Although smaller than the ~5 fold contribution seen in Brf1 supplemented whole cell extracts (comparing appropriate lanes between Figs. 2A and B), this result nonetheless confirms that Brf1 is targeted for repression in extracts of CPZ-treated cells.

**Brf1 rescues the TFIIIB-DNA complex assembly defect in CPZ-treated cell extracts**

Previously we showed that the formation of heparin-resistant TFIIIB-DNA complexes on a tRNA gene template is severely impaired in CPZ-treated versus control cell extracts (17) and Fig. 2D). Since the preceding experiments established that the transcriptional activity of Brf1 is repressed in these extracts, we expected that Brf1 supplementation of the CPZ-treated extract would restore TFIIIB-DNA complex assembly in the same manner as it had restored transcription (Fig. 2B). Indeed, supplementation with individual TFIIIB subunits or combinations of these factors resulted in large increases in complex formation, which paralleled the changes in transcription (compare Figs. 2B and 2D). As before, only reactions containing Brf1 rescued the complex assembly defect; TBP and Bdp1 by themselves had no effect. Consistent with the
transcription data (Fig. 2A), supplementation of the control extract produced only small increases in TFIIIB-DNA complex assembly; the largest effect (2.5 fold) was achieved when all three subunits were added\(^2\). The results from both transcription and complex assembly assays show that the differential between the extracts is minimized but not eliminated following Brf1 supplementation. Thus, in addition to affecting Brf1 function, the data suggest that some other step(s) and/or factor may be affected under repressing conditions.

**CPZ treatment reduces polymerase occupancy on tRNA genes in a Maf1-dependent manner**

The inhibition of TFIIIB-DNA complex assembly mediated, at least in part, by changes in Brf1 activity provides one mechanism for repressing pol III transcription. However, given the rapid kinetics of repression (e.g. \(t_{1/2} < 10\) min for CPZ, (17), and the high stability of TFIIIB-DNA complexes (19;21), we were interested to know what changes might occur to transcription complexes already assembled on the DNA when repressing conditions are imposed: Are they disrupted or is their ability to direct transcription inhibited by a post-TFIIIB assembly mechanism? To address these issues, chromatin immunoprecipitation (ChIP) was used to examine the occupancy of representative TFIIIC, TFIIIB and polymerase subunits on pol III genes before and after treatment with CPZ. One subunit of TFIIIC (Tfc1), two subunits of TFIIIB (Brf1 and Bdp1) and two pol III subunits (Rpc82 and Rpc53), each tagged with a triple HA epitope, were analyzed for their occupancy on three different tRNA genes. Cells were crosslinked with formaldehyde either before or 1 hour after CPZ-treatment (when maximal repression has been achieved, (17) and the proteins were immunoprecipitated with an anti-HA antibody to enrich the set of DNA fragments
associated with each protein. Multiplex PCR of immunoprecipitated and input DNA samples was then performed for each gene of interest and two negative controls (a ribosomal protein gene, TCM1 and an intergenic region on chromosome V, Int V) under conditions where product yield was linearly dependent on the amount of each sample used for PCR. A representative experiment is shown in Fig. 3. Following normalization for input DNA and data averaging over multiple experiments, we determined that transcriptional repression by CPZ did not detectably affect the occupancy of Tfc1 and produced only a small effect on Brf1 and Bdp1 (reduced 1.7 ± 0.4 fold compared to the untreated control, Fig. 3A). However, a more substantial effect was observed on polymerase occupancy, which was reduced 6 ± 1 fold compared to the untreated control. (Fig. 3B). Importantly, the reduced occupancy of pol III on tRNA genes was not observed in a maf1Δ strain following CPZ treatment (Fig. 3B). Since maf1Δ strains are completely refractory to repression by CPZ (17), this result serves to demonstrate the specificity of the reduction in polymerase occupancy in the wild-type MAF1 strain. Thus, together with the preceding biochemical data (Figs. 1 and 2 and (17), we conclude that repression by CPZ (and presumably other Maf1-mediated repression) is achieved by affecting two steps in transcription: (i) The assembly of new TFIIIB-DNA complexes and (ii) the recruitment of pol III to preexisting TFIIIB-DNA complexes. Finally, we note that attempts to ChIP myc-tagged Maf1 in normal and CPZ-treated cells have not produced signals above the non-specific controls despite extended (overnight) formaldehyde crosslinking and the inclusion of a protein-protein crosslinker, dimethyl adipimidate. While these results do not exclude the possibility that Maf1
may be associated (e.g. transiently) with pol III genes, there is currently no biochemical (see below) or in vivo support for a direct or indirect interaction of Maf1 with DNA.

**Recombinant Maf1 inhibits pol III transcription in vitro**

In order to explore the biochemical function of Maf1 we have expressed full-length and/or truncated forms of the protein from *S. cerevisiae* and *S. pombe* in bacteria. Maf1 contains three phylogenetically conserved sequence blocks (A, B and C) but the protein from *S. cerevisiae* contains an additional non-conserved domain between blocks A and B that gives it a molecular mass of 45 kDa versus 25-29 kDa for most other species. Full-length *S. pombe* Maf1 was purified either as a glutathione-S-transferase (GST) fusion protein (GST-*Sp* Maf1) or with a C-terminal histidine tag (*Sp* Maf1). The latter strategy was also used to purify fragments of *S. cerevisiae* Maf1 containing either domain A (ScMaf1(A)) or domains B and C (ScMaf1(B+C)). In all cases, the recombinant proteins were determined to be at least 85% pure by SDS-PAGE. The proteins were then assayed to determine if they could function as inhibitors of pol III transcription. Varying amounts of the different Maf1 proteins were combined with rBrf1, rTBP, yeast Bdp1, TFIIC and pol III fractions before addition of a tRNA gene template and nucleotides to start transcription. ScMaf1(A) and two control proteins (GST or BSA) did not significantly affect transcription under these conditions (Fig. 4A and data not shown). However, ScMaf1(B+C) and both of the full-length SpMaf1 proteins were potent inhibitors of transcription; at the upper end of the titration, transcription was reduced to only a few percent of the levels achieved in reactions lacking Maf1 (Fig. 4A). Similar results were obtained in assays of TFIIC-
independent \textit{SNR6} transcription when Maf1 was combined with TFIIIB subunits and pol III prior to addition of the template (Fig. 4C, six leftmost lanes).

Based on the reduced occupancy of pol III on tRNA genes under repressing conditions (Fig. 3B) and the known interaction of Maf1 and pol III (16) we next examined whether Maf1 could inhibit transcription from pre-assembled initiation complexes. The assembly of TFIIIB on a tRNA gene in the presence of TFIIIC and on the \textit{SNR6} gene in the absence of TFIIIC was allowed to proceed to equilibrium during a 1 hour incubation prior to the addition of the different Maf1 proteins, pol III and NTPs. As before, \textit{Sc}Maf1(A) and GST did not affect the level of transcription from either template (Figs. 4B and 4C). Conversely, the \textit{Sc}Maf1(B+C) fragment and \textit{Sp}Maf1 were still effective inhibitors of tRNA and \textit{SNR6} transcription although higher amounts of these proteins were required to achieve the same level of inhibition as reactions where TFIIIB complexes were not pre-assembled (compare lanes in Fig. 4B with the corresponding lanes in Fig. 4A and the before and after assembly lanes in Fig. 4C). In addition to demonstrating that the inhibition of transcription by Maf1 in vitro is not dependent on TFIIIC (Fig. 4C), the different concentration dependence of inhibition before and after complex assembly implies that Maf1 affects multiple steps in transcription. One of the affected steps is likely to be polymerase recruitment/recycling. This conclusion is based on the inhibition of transcription after TFIIIB assembly (Fig. 4B and 4C), the direct interaction between Maf1 and pol III (16) and the reduced occupancy of pol III on tRNA genes under repressing conditions (Fig. 3). Additional support for this conclusion is described below.
Maf1 binding to Brf1 blocks TFIIIB complex assembly

Since whole cell extracts from CPZ-treated cells are defective in the assembly of TFIIIB (17), we examined whether recombinant Maf1 could block TFIIIC-directed assembly of recombinant TFIIIB subunits on a tRNA gene by native gel electrophoresis. ScMaf1(B+C), SpMaf1 and GST-SpMaf1, but not GST alone, were potent inhibitors of TFIIIB complex assembly (Fig. 5A).

In contrast, the Maf1 proteins did not detectably affect TFIIIC-DNA interactions, bind to TFIIIC-DNA complexes or have intrinsic DNA binding activity under the conditions employed (Fig. 5A and data not shown). The robust effect of Maf1 in these assays, which employed relatively modest amounts of the protein, suggests that inhibition of complex assembly accounts for much of the effect of rMaf1 in transcription assays when complexes are not preassembled (Figs. 4A and 4C).

Next we examined whether rMaf1 was able to disrupt preassembled TFIIIB-TFIIIC-DNA complexes. Addition of the ScMaf1(B+C) fragment or SpMaf1 to TFIIIB complex assembly reactions that had reached binding equilibrium, followed by a further incubation before gel electrophoresis, did not have any effect on the amount of complex (Fig. 5B and data not shown). This finding supports the conclusion that the inhibitory effect of rMaf1 on transcription from preassembled complexes (Fig. 4B and 4C) involves a post-TFIIIB recruitment step.

The first step in the assembly of TFIIIB on a tRNA gene is the recruitment of Brf1 by promoter-bound TFIIIC (reviewed in (29). The ability of Maf1 to block this limiting step in complex assembly was assessed by adding Brf1 to mixtures containing Maf1 proteins and
TFIIIC-DNA. As for the assembly of TFIIIB, Maf1 inhibition of Brf1 recruitment onto TFIIIC-DNA complexes was complete and specific (Fig. 5C). Moreover, domains B+C of Maf1 but not domain A, were functionally important in this assay in agreement with the transcription data (Fig. 4A and 4B). Given that the effects of Maf1 on transcription are independent of TFIIIC (Fig. 4C), the preceding results suggest that Maf1 interacts directly with Brf1. This possibility was tested in pull-down assays using recombinant Brf1 bound to Ni²⁺-NTA-resin. \[^{35}\text{S}]\)-Methionine-labeled \textit{S. cerevisiae} and \textit{S. pombe} Maf1 were incubated with resin alone or Brf1–resin under stringent ionic conditions to prevent non-specific binding. After extensive washing, the bound proteins were analyzed by SDS-PAGE and autoradiography (Fig. 5D). Maf1 bound to the Brf1-resin but not to the control resin indicating a weak but specific interaction between the two proteins under these conditions.

**Repression by CPZ does not Quantitatively Affect the Association of Maf1 with Brf1 or pol III.**

From the above experiments, the inhibitory function of Maf1 under in vitro conditions seemed likely to involve its stoichiometric binding to Brf1 (and presumably to pol III). To test whether this mechanism of repression is observed in yeast cells and to confirm the Maf1-Brf1 interaction in a more physiological context, we performed co-immunoprecipitation experiments with control and CPZ-treated extracts prepared from strains bearing HA-tagged Brf1 or Rpc82 and/or myc-tagged Maf1. Importantly, the presence of the tag on these proteins did not affect repression by CPZ as determined by Northern analysis\(^3\). Each monoclonal antibody specifically precipitated the corresponding tagged protein and reduced its level in the resulting supernatants.
by 85-90% (Fig. 6)³. In the doubly-tagged Brf1-HA, Maf1-myc extract, co-immunoprecipitation of both proteins was observed although only a small fraction of the total amount of Maf1 and Brf1 (<1%) was recovered in the Brf1-HA and Maf1-myc precipitates, respectively. A somewhat larger fraction of both Maf1 and pol III was found in co-immunoprecipitates from the Rpc82-HA, Maf1-myc extracts (~5-10%, Fig. 6B), consistent with previous findings (16). Interestingly, a comparison of the amount of Brf1, pol III and Maf1 in co-immunoprecipitates from control and CPZ-treated extracts showed no significant changes (Fig. 6). Since Western blotting of the various extracts and total cell lysates showed that Maf1, Brf1 and pol III protein levels do not change significantly under the repressing conditions employed here (Fig. 6, supplementary Fig. S1B, (17), the co-immunoprecipitation data indicate that the mechanism of repression by Maf1 in vivo does not involve a quantitative change in its interaction with either Brf1 or pol III. Consistent with this conclusion, over-expression of Maf1 (>10-fold) in yeast did not reduce the level of pre-tRNALeu, pre-tRNAIle, pre-tRNAlys or pre-tRNAHis in early to mid log phase (<2 x 10⁷ cells/ml) under otherwise normal growth conditions (supplementary Fig. S1A). Moreover, in contrast to the excess molar amounts of rMaf1 that were used to inhibit transcription and complex assembly with purified components (Figs. 4 and 5), quantitative Western blotting (23) revealed that control and CPZ-treated yeast whole cell extracts contain only about one tenth the amount of Maf1 (0.5-1.0 fmole/µg protein) relative to Brf1 (5-10 fmole/µg protein). These data all support a non-stoichiometric mechanism of
inhibition by Maf1.

**DISCUSSION**

In this study, we have shown that Maf1-dependent repression of pol III transcription involves inhibitory effects on two distinct steps: (i) de novo assembly of the initiation factor TFIIIB onto DNA and (ii) recruitment of pol III to TFIIIB-DNA complexes that are already assembled when repressing conditions are encountered. The inhibition of these steps was determined using CPZ, an anti-fungal compound, to induce repression. However, the Maf1-dependence of both of these steps (Fig. 3 and (17)) indicates that the underlying mechanism(s) will apply to many other, if not all, repressing conditions in yeast. Indeed, we have yet to find a condition that represses pol III transcription in a manner that is not dependent on Maf1. In addition to the down-regulation that occurs normally during the yeast growth cycle and repression induced by secretory defects, DNA damage and various drug treatments (e.g. tunicamycin, rapamycin, (17)), we have found that carbon source starvation, ER stress (5 mM DTT) and oxidative stress (0.5 mM hydrogen peroxide) conditions also require Maf1 in order to achieve repression of pol III transcription (supplementary Fig. S2).

Pol III transcription is strongly repressed in whole cell extracts prepared from CPZ-treated cells. As noted previously, this in vitro repression is absolutely dependent on Maf1 and is due in large part to the inhibition of TFIIIB complex assembly (17). In agreement with these findings, we have found that the Brf1 subunit of TFIIIB is an important target of Maf1-dependent transcriptional repression: The transcription activity of Brf1 purified from CPZ-
treated extracts was reduced about 3-fold relative to the untreated control (Fig. 1C) and this effect compares favorably with the ability of rBrf1 to minimize the differences in transcription (from 15 fold to ~3 fold, Fig. 2) and TFIIIB-complex formation in the whole cell extracts. Importantly, Brf1 was the only component of the pol III transcription apparatus to elicit these effects (Fig. 2, (17)². Thus, we conclude that a defect in the complex assembly function of Brf1 is one cause of pol III transcriptional repression. Consistent with this conclusion and the conservation of Maf1-dependent repression mechanisms, TFIIIB has been identified as a likely target of repression by rapamycin treatment and DNA damage in yeast (13;15).

CPZ treatment also caused a marked Maf1-dependent reduction in the occupancy of tRNA genes by pol III (Fig. 3). This effect, as well as the more modest reduction in the occupancy of TFIIIB subunits (Brf1 and Bdp1), appears to be a characteristic response of the pol III machinery to repressing conditions, as these changes have been reported for cells in stationary phase (30;31) and after carbon source starvation (>25 minutes, (32). The kinetics of repression following carbon source starvation and CPZ treatment, as well as other repressing conditions, is very rapid (t½ < 10 minutes, (17;32). Accordingly, the maintenance of TFIIIB-DNA complexes in cells at early times following the induction of repression and their relative persistence at late times (Fig. 3, (31;32) demonstrates the importance of interrupting pol III recruitment/recycling for efficient transcriptional repression. Thus, it seems that control of pol III occupancy on the DNA is a general and critical Maf1-dependent regulatory mechanism.

The biochemical effects of recombinant Maf1 on pre- and post-TFIIIB assembly steps
(Figs 4 and 5) support the findings obtained from CPZ-treated yeast cell extracts and ChIP experiments with respect to the repression of two distinct steps in transcription. The ability of rMaf1 to inhibit Brf1 binding to TFIIIC-DNA (Fig. 5C), presumably due to its direct interaction with Brf1 (Fig. 5D) is consistent with the reduced transcription activity of Brf1 and the ability of rBrf1 to largely rescue the transcription and complex assembly defects in the CPZ-treated extract (Figs. 1 and 2). Similarly, the inability of rMaf1 to disrupt pre-assembled TFIIIB-DNA complexes and yet inhibit transcription from these complexes (Figs. 4 and 5), coupled with its direct interaction with pol III (16), appears to explain the reduced occupancy of the polymerase on tRNA genes under repressing conditions. However, the relative amounts of rMaf1 required to achieve these effects are significantly greater than those found in yeast extracts (see Results section and Figs 4-6). This may indicate that the activity of rMaf1 is lower than yeast Maf1 from repressed cell extracts (perhaps because of missing posttranslational modifications) and/or that the reconstituted system is missing components required for efficient Maf1-dependent repression. In any event, the sub-stoichiometric amounts of Maf1 in our yeast extracts (relative to Brf1), the low amounts of Brf1 and pol III associated with Maf1 (Fig. 6) and the fact that no quantitative changes in these associations occur in repressed cell extracts provide compelling evidence that the biologically relevant mechanism of repression by Maf1 does not involve its stoichiometric binding to these components. Conversely, we suggest that the repression of complex assembly and transcription in our purified system by excess molar amounts of rMaf1 is achieved though physiologically important interactions with Brf1 and pol III but involves a non-
phyisologic, stoichiometric binding mechanism.

By excluding a stoichiometric mechanism of repression in yeast (Fig. 6), our data implicate Maf1, either directly or indirectly, in a catalytic process that inhibits TFIIB-DNA complex assembly and transcription. The nature of this catalytic process remains a subject for further study. However, given the available data, a reasonable possibility is that repressing conditions affect the activity of pol III and/or Brf1 via covalent modifications that are dependent on the interactions of these proteins with Maf1. Although regulatory modifications to yeast Brf1 have not been reported, the function of Brf1 in human cells can be both activated and inhibited by phosphorylation (7-9). Specific subunits of pol III are also phosphorylated (33) and several subunits have recently been found to be sumolyated (34;35). While the functional and/or regulatory significance of these changes to pol III is presently unknown, their importance for repression cannot be discounted by our finding that partially purified pol III from CPZ-treated extracts was active in transcription (Fig. 1B). For example, we cannot exclude the possibility that inactivating modifications may have been removed during the purification. Thus, Maf1-dependent regulation of pol III activity remains a viable explanation for the reduced occupancy of the polymerase on tRNA genes under repressing conditions.

ACKNOWLEDGEMENTS

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Transcriptional Regulation, Cold Spring Harbor Laboratory Press,


FOOTNOTES

1. see the Saccharomyces Genome Database, [http://www.yeastgenome.org/](http://www.yeastgenome.org/)
2. J. Lee, unpublished data.
3. N. Desai, unpublished data.

FIGURE LEGENDS

**Fig. 1** Reconstitution of transcription with TFIIIC, pol III and TFIIIB fractions from control and CPZ-treated cells. (A) TFIIIC purified from control and CPZ-treated extracts has similar transcription activity. Multiple-round transcription on a tRNA^Leu^ template was reconstituted with an empirically determined excess of TFIIIB components (rBrf1 10 pmole, rTBP 1 pmole, affinity-purified yeast Bdp1 280 fmole, and polymerase III, 0.75 µg). Reactions contained varying amounts of either CPZ-treated or control TFIIIC as indicated. Immunoblotting using an anti-Tfc4 antibody (lower panel) shows that the fractions contained equivalent amounts of TFIIIC. (B) Pol III purified from control and CPZ-treated extracts has similar transcription activity. Transcription was reconstituted as in panel A using equal amounts of TFIIIC (50 fmole) and varying amounts of polymerase. Immunoblotting using an anti-Rpc34 antibody (lower panel) shows that the fractions contained equivalent amounts of pol III. (C) TFIIIB purified from CPZ-treated extracts has lower transcription activity. Reactions were performed as in panel A except that equal amounts of TFIIIC (50 fmole) and polymerase (0.5 µg) and varying amounts of TFIIIB purified from either CPZ-treated or control extracts were used.
Immunoblotting using an anti-Brf1 antibody (lower panel) shows that the CPZ-treated TFIIIB fraction has ~6 fold lower levels of Brf1 than the control TFIIIB fraction. (D) The activity of Brf1 is reduced by CPZ treatment. The data in panel C are plotted as a function of the absolute amount of Brf1 in each reaction.

Fig. 2. Supplementation of control and CPZ-treated whole cell extracts with TFIIIB subunits.

(A) The effect of supplementing a control whole cell extract (50 µg protein) with recombinant TFIIIB subunits (TBP, 1 pmole; Brf1, 10 pmole; Bdp1, 280 fmole) was assessed in a multiple round transcription assay using a tRNA^Leu template. (B) The experiment in panel A was performed in parallel with a CPZ-treated whole cell extract (50 µg protein) that had been prepared together with the control. The gel images shown in panels A and B were collected together using a single phosphor storage screen. The transcription activity of the reactions is indicated under each lane and is expressed relative to the unsupplemented CPZ-treated extract.

(C) Supplementation of Brf1-depleted whole cell extracts with rBrf1 reduces the transcription differential. Mock-depleted and Brf1-depleted whole cell extracts were supplemented with rTBP and rBrf1 as indicated. The difference in transcription between equivalent reactions containing control and CPZ-treated extracts is indicated under the control lanes. (D) Brf1 supplementation can restore TFIIIB-DNA complex assembly to CPZ-treated extracts. Heparin-resistant TFIIIB-DNA complex formation on a tRNA gene template was assayed by native gel electrophoresis. The indicated amounts of recombinant TFIIIB subunits were added to CPZ-
treated extract (40 µg protein). Complex assembly with an equivalent amount of control extract (Con) is shown in the leftmost lane. The arrow shows the position of the TFIIIB-DNA complex.

Fig. 3. Chromatin immunoprecipitations from control and CPZ-treated cells. Fractions used for immunoprecipitation (Input) of control and CPZ-treated strains were titrated in multiplex PCR reactions containing primer pairs for a ribosomal protein gene (*TCM1*), a tRNA*Leu* gene (*SUP54*) and an intergenic region from chromosome V (Int V). PCR reactions of the corresponding immunoprecipitated DNAs (IP) are shown in the rightmost lanes of each panel (titrations of these DNAs were performed in separate experiments). The HA-tagged strains are indicated below each panel. (A) TFIIIC and TFIIIB occupancy of the *SUP54* tRNA*Leu* gene does not change significantly after CPZ treatment. Equivalent results were obtained for tRNA*Lys* and tRNA*Tyr* (data not shown). (B) Pol III occupancy of the *SUP54* tRNA*Leu* gene is reduced in a Maf1-dependent manner after CPZ treatment. Chromatin immunoprecipitation of Rpc82-HA3 was performed in *MAF1* wild-type and *maf1Δ* strains as indicated. An untagged *maf1Δ* strain was used as a control (first three Input lanes and first IP lane). Equivalent results were obtained in an Rpc53-HA3 strain (data not shown).

Fig. 4. Recombinant Maf1 inhibits TFIIIC-dependent and TFIIIC-independent pol III transcription. (A) Multiple round transcription on a tRNA*Leu* gene was carried out in a
reconstituted system (see Materials and Methods) with rMaf1 proteins added prior to complex assembly. **(B)** Reaction conditions were the same as in panel A except that TFIIIB-TFIIIC-DNA complexes were preassembled before the addition of rMaf1, pol III and nucleotides. **(C)** Multiple round transcription on the U6ΔB template was carried out in a reconstituted system (see Materials and Methods) with rMaf1 proteins added either prior to or after TFIIIB-DNA complex assembly, as indicated. Lanes labeled “G” contained GST (4 µg in lane 4 and 10 µg in lane 12) as a negative control. Relative transcription is indicated under each panel.

Fig. 5. Maf1 binding to Brf1 blocks the assembly of TFIIIB onto DNA.

**(A)** Maf1 inhibits TFIIIB assembly onto TFIIIC-DNA complexes. TFIIIC-dependent assembly of recombinant TFIIIB subunits (rBrf1 2 pmole, rTBP 25 fmole, Bdp1 200 fmole) on a tRNA gene was performed in the presence of different recombinant Maf1 proteins or a GST control protein. The complexes were resolved on a native 4% polyacrylamide gel. **(B)** Maf1 does not dissociate TFIIIB-TFIIIC-DNA complexes. ScMaf1(B+C) was incubated with TFIIIB subunits as in panel A or added to pre-assembled TFIIIB-TFIIIC-DNA complexes for 20 min. prior to gel analysis. **(C)** Maf1 blocks Brf1 binding to TFIIIC-DNA complexes. rBrf1 (8 pmole) was added to pre-formed TFIIIC-DNA complexes in the presence of different amounts of ScMaf1(A), GST, ScMaf1(B+C) or SpMaf1. **(D)** Maf1 binds to Brf1. [35S]-labeled Maf1 proteins were incubated with resin alone or Brf1-resin, washed in buffer containing 400 mM NaCl and the bound protein was eluted and analyzed by SDS-PAGE and autoradiography. 10% of the
input was also loaded.

**Fig. 6. Interactions of Maf1 with Brf1 and Pol III in control and CPZ-treated whole cell extracts.** Immunoprecipitation and Western analysis was performed with antibodies to HA and myc epitopes using extracts containing the indicated tagged proteins. The antibodies used for precipitation and blotting are shown on the left and right side of each panel, respectively. Control (-) and CPZ (+) samples are indicated above each lane. Input lanes contain 10-20 µg of the extracts used for immunoprecipitation. (A) Co-immunoprecipitation of Maf1 and Brf1. Precipitates from 2 mg of extract were eluted in SDS sample buffer (35 µl). Myc and HA IP lanes that were probed with the precipitating antibody contained 5 µl and 10 µl of the eluate, respectively. IP lanes probed for the co-immunoprecipitating protein contained 30 µl of the eluate. (B) Co-immunoprecipitation of Maf1 and pol III. Precipitates from 2 mg of extract were eluted as in panel A. All IP lanes contain 5 µl of the eluted sample.
Fig. 2

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C. Control

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**α HA**

**Rpd32**

**Maf1**

**α myc**
Supplementary Figure S1. Over-expression of Maf1 does not reduce the level of pre-tRNA^{Leu}, pre-tRNA^{Ile}, pre-tRNA^{Lys} or pre-tRNA^{His} in mid log phase cells (A600 <0.7). (A) Northern analysis of precursor tRNAs and U4 snRNA in a maf1Δ strain containing the centromeric vector pRS314 without or with ScMaf1 (pRS314+Maf1) or a 2-micron Maf1-containing plasmid (pRS426+Maf1). Western analysis showing the expression of Maf1 in the three strains is shown in the lower panel. Whole cell lysates were probed with a rabbit polyclonal antibody raised against recombinant ScMaf1(B+C). (B) Repression of pol III transcription by chlorpromazine does not affect the amount or mobility of Maf1. Maf1 was tagged at its C-terminus with a triple HA epitope in strain W303. Western analysis was carried out using whole cell lysates prepared from the tagged and untagged strains before and after CPZ treatment for one hour.
Supplementary Figure S2

Supplementary Figure S2. Maf1 is required for repression of pol III transcription in response to ER stress (panel A), oxidative stress (panel B) and carbon source starvation (panel C). Wild type strain (W303α) and its maf1Δ derivative were grown to mid-log phase (A600 0.5-1.0) in synthetic complete medium (panels A and B) prior to addition of DTT (5 mM, Travers et al., 2000 Cell, 101, 249-258) or hydrogen peroxide (0.5 mM), respectively. Strain 1608-21C (Oehlen and Cross, 1994 Genes Dev., 8, 1058-1070) and its maf1Δ derivative were grown to mid-log phase (A600 0.5-1.0) in YP-Galactose. The cells were harvested and resuspended in YP-Raffinose, which does not support growth in these strains. Aliquots were harvested at the indicated times and total RNA was prepared for Northern analysis.
Supplementary Data and Methods

Chromatin Immunoprecipitation and Quantitative PCR.

CPZ (250 µM) was added to logarithmically-growing yeast strains (A600 0.6-0.7). After 1 hr, treated and untreated cells were cross-linked with formaldehyde (final concentration 1%) at 20°C for 20 min, harvested and broken with chilled Silica-Zirconia beads (0.5 mm dia.) using a Minibeadbeater-8 (Biospec Products) at 4°C. The sonicated chromatin pellet was clarified by centrifugation and stored at -70°C. Monoclonal anti-HA antibody (2µg, Roche Biochemicals) was added to the chromatin fraction (400 µl) and was incubated overnight at 4°C, followed by a 3 hour incubation with Protein A-Sepharose (30 µl). After extensive washing, the immunoprecipitated protein with cross-linked DNA was eluted twice with TE-SDS buffer (75 µl). The eluates were incubated overnight at 65°C to reverse the cross-linking and digested with proteinase K and RNase A (Kuras and Struhl, 1999 Nature, 399, 609-613). Immunoprecipitated DNA was purified using Quiaquick PCR purification columns and eluted in 10 mM Tris-HCl buffer, pH 8.0 (50 µl). Input DNA was prepared in the same way without the immunoprecipitation step.

DNA samples were analyzed by quantitative PCR using primer pairs for three different tRNA genes and two negative controls (a ribosomal protein gene, TCM1 and an intergenic region on chromosome V, Int V). PCR (25 µl) contained varying amounts template, 12.5 pmole of each primer, 0.1 mM dNTPs, [32P]-dATP (2.5 µCi) and Platinum Taq polymerase (0.5 Units, Invitrogen). Labeled products were separated on 10% polyacrylamide-1X TBE gels and were quantified by phosphorimager analysis. Beads-only control DNA was prepared in parallel with immunoprecipitated samples by omitting the anti-HA antibody. Background PCR signals were also tested with DNA from the untagged parent strain.
The primers used to amplify tRNA genes for ChIP experiments are as follows:

**Leucine** tRNA\(_{\text{tL}(CA)G2}\) on chromosome VII

F 5’-AGACGAGCAGCTTATCCCATAATGA-3’
R 5’-GTGACGCCTGGTGCGTAAAAGAT-3’

**Tyrosine** tRNA\(_{\text{tY}(GA)G2}\) on chromosome X

F 5’-TTCACTCTGAACCACAGCTTGGAAGGA-3’
R 5’-CAAGTCTGGGAAAGTGGAATGGAGACA-3’

**Lysine** tRNA\(_{\text{tK}(UU)G1}\) on chromosome VII

F 5’-GTTTACCCTTCATATACATGTGCATT-3’
R 5’-AATCTTGGAAAAATAGTGAAACCGGG-3’

**TCM1**

F 5’-GTCGAAGCTGTCCATCCGTCTGGAC-3’
R 5’-GTTCAAAAACGCTGTCGACAGCAAC-3’

**Intergenic region on chromosome V** (Komarnitsky et al., 2000 *Genes Dev.*, 14, 2452-2460)

F 5’-GGCTGTCAGAATATGGGGCCGTAGTA-3’
R 5’-CACCCCGAAGCTGCTTTCACAATAC-3’

The sequences of primers used to amplify the cDNA for *S. pombe* MafI from pGAD GH (Stratagene) are:

5’-CGCTCTAGAACTAGTGGATCCC-3’
5’-TCAAGTCTCGAGATCATCCATGTTAGCACAACCAATC-3’
Two steps in Maf1-dependent repression of transcription by RNA polymerase III
Neelam Desai, JaeHoon Lee, Rajendra Upadhya, Yaya Chu, Robyn D. Moir and Ian M. Willis

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