Two Steps in Maf1-dependent Repression of Transcription by RNA Polymerase III*[S]

Neelam Desai‡, JaeHoon Lee‡, Rajendra Upadhya, Yaya Chu, Robyn D. Moir, and Ian M. Willis§

From the Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461

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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 transcription factor (TF) IIIB onto DNA as well as the recruitment of pol III to preassembled 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.

Transcription of the large rRNAs by RNA polymerase (pol) I and of 5 S rRNA and tRNAs by pol III is tightly co-regulated under essentially all conditions (1–3). This coordinate 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 and 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 extracellular signal-regulated kinase, casein kinase II, 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 Tdp3, a regulatory subunit of protein phosphatase 2A (12), as well as casein kinase II, which is necessary for efficient TFIIIB-DNA complex assembly (13). Casein kinase II copurifies with the TBP and Brf1 subunits of the initiation factor TFIIIB and phosphorylates TBP (most likely on Ser-128 (13)). Yeast TFIIIB (which comprises TBP, Brf1, and Bdp1 polypeptides) and in some cases pol III itself have been suggested as regulatory targets based on their 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 antifungal compound chlorpromazine (CPZ). As these conditions are known to activate at least three distinct signaling pathways (secretory signaling, target of rapamycin (TOR) kinase, and DNA damage response pathways), it is evident that these pathways must converge upon Maf1 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...
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In this work, we examined the consequences of Maf1-dependent repression in vivo and in vitro following CPZ treatment. We found that repression is affected at two different steps in transcription, namely TFIIIB-dependent transcription and polymerase recruitment. Maf1 was shown to interact with a small fraction of the Brf1 and pol III in control yeast extracts, and these interactions were 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 W303a (MATa leu2-3,112 trp1-1 ura3-1 can1-100 ade2-1 his3-11,15 sds-1-1). Hemagglutinin (HA) or Myc tags were added to the 3’-ends of the TFC1, BRF1, BDP1, RPC53, RPC82, and MAF1 genes2 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 (17). Extracts were fractionally fractionated on a ReX70 to obtain the Brf1 fraction (19). Subsequent chromatography of the Brf1 fraction on DEAE-Sephadex A-25 (19) yielded a flow-through fraction containing TFIIIB. The column was then developed with a 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 (Ref. 21 and references therein). TBP was kindly provided by Dr. Michael Brenowitz. Yeast Bdp1 with a histidine tag at its C terminus was purified from the Brf1 fraction (19) under denaturing conditions on Ni2+-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 MgCl2, 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 (18, 19).

Maf1 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 Bio-Rad 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 Ni2+-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 phenylmethylsulfonfonyl fluoride, leupeptin, pepstatin, and aprotinin at a concentration of 5 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 (SMLNT® coupled reticulocyte lysate system, Promega) to generate 35S-labeled proteins. Each pull-down reaction (100 μl) contained control or Brf1 (100 pmol)-resin (10 μl) and 35S-labeled Maf1 (10 μl) in binding buffer (see above) containing 10 mM imidazole, 5 μg/ml bovine serum albumin, and 0.1% Triton X-100. After incubation at 4 °C for 2 h, the resins were washed in binding buffer containing 20 mM imidazole (3 × 20 min) and then in binding buffer containing 30 mM imidazole, 400 mM NaCl, and 0.2% Triton X-100 (3 × 20 min). Samples were boiled in Laemmli buffer and 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 tRNA47-end template (YPEp13, 0.5 μg) as described previously (24). Transcription of the B box-deleted SNR6 template (U6&8) was performed similarly except that the extracts were supplemented with rTBP (20 pmol). To prepare Brf1-immunodepleted extracts, whole cell extracts (400 μg) were incubated with an affinity-purified Brf1 polyclonal antibody at 4 °C for 1 h. Brf1(100 pmol) at 4 °C for 1 h. Brf1 (100 pmol) was preincubated with preassembled TFIIIB and rTBP (20 pmol) for 30 min, washed in preincubation buffer, and then incubated with rTBP (20 pmol) 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, TFIIH-TFIIID-DNA, and Brf1-TFII-ID-CDNA complexes were assembled on a sup3-eST tRNA gene and resolved on native 4% polyacrylamide gels as described previously (17, 21). Immunoprecipitation of rMaf1 fragments with preassembled TFIIID IC-DNA complexes was carried out for an additional 20 min prior to gel loading. rMaf1 polypeptides were diazoylated in complex assembly buffer for these reactions (25).

Immunoprecipitation of Maf1, Brf1, 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 Applied Science) in KBC100 buffer (see above) in a total volume of 400 μl at 4 °C for 3–4 h (Rcp82 and Maf1) or overnight (Brf1) followed by a 3-h incubation with protein G-Sepharose beads (20 μl). The beads were washed with KBC100 buffer (3 × 15 min) and boiled in SDS sample buffer, and aliquots were subjected to immunoblotting.

Chromatin Immunoprecipitation and Quantitative PCR—Chromatin immunoprecipitation 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 Supplemental 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 with untreated cell extracts. The low transcription activity of

2 See the Saccharomyces Genome Database (www.yeastgenome.org).
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 TFIIIB substantially rescued the transcriptional defect, while excess molar amounts of the assembly factor TFIIIC or pol III from control extracts had no effect. To further examine the effect of CPZ treatment in this system, TFIIIB, TFIIIC, and pol III were partially purified from the control and treated extracts to compare their individual activities in transcription. Western blotting for representative TFIIIC and pol III subunits indicated that the corresponding control and treated fractions contained equivalent amounts of these factors per microgram of protein (Fig. 1, A and B, Tfc4 and Rpc34), consistent with their levels in the unfractionated extracts (17). Subsequent titrations of the TFIIIC or pol III fractions under conditions in which these components are limiting for transcription revealed no significant differences in their activity (Fig. 1, A 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 of Maf1/2.5 μg of protein). For TFIIIC, 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 TFIIIC-independent (27), yet the large differential in transcription between the control and treated extracts was retained. On the basis of these findings, a direct role for TFIIIC in the repression of pol III transcription by CPZ seems unlikely.

In contrast to the results described above for TFIIIC and pol III, the TFIIIB 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 6-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 was 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).a Supplementation of a control extract with excess molar amounts (≥3-fold) of rTBP, rBrf1, or affinity-purified yeast Bdp1 resulted in negligible to small effects on transcription that 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 three TFIIIB 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- to 2.5-fold) but does not com-

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a J. Lee, unpublished data.
indicated amounts of recombinant TFIIIB subunits were added to CPZ-treated extract (40 μg of protein) with recombinant TFIIIB subunits (TBP, 1 pmol; Brf1, 10 pmol; Bdp1, 280 fmol) was assessed in a multiple round transcription assay using a tRNA^tRNA template. B, the experiment in A was performed in parallel with a CPZ-treated whole cell extract (50 μg of protein) that had been prepared together with the control. The gel images shown in 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 each lane. 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 of 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.

Comparative 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. 2, A and B), the transcription differential in the mock-depleted control and CPZ-treated extracts was reduced from 12-fold (Fig. 2C, lanes 1 and 6) to 2.5-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. 2, A and B) 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 Fig. 2, A 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 (Ref. 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 Fig. 2, B and D). 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.3 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. 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 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 TFIIC (Tfc1), two subunits of TFIIIB (Brf1 and Bdp1), and two pol III subunits (Rp62 and Rp53), each tagged with a triple HA epitope, were analyzed for their occupancy on three different tRNA genes. Cells were cross-linked with formaldehyde either before or 1 h 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-fold) with rMaf1 proteins added prior to or after 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 “Experimental Procedures”) 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.

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—to explore the biochemical function of Maf1 we 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 B and C that gives it a molecular mass of 45 versus 25–29 kDa for most other species. Full-length S. pombe Maf1 was purified either as a GST fusion protein (GST-SmMaf1) or with a C-terminal histidine tag (SmMaf1).

The latter strategy was also used to purify fragments of S. cerevisiae Maf1 containing either domain A (SmMaf1(A)) or domains B and C (SmMaf1(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 whether they could function as inhibitors of pol III transcription. Varying amounts of the different Maf1 proteins were combined with rBrf1, rTBP, yeast Bdp1, TFIIIC, and pol III fractions before addition of a tRNA gene template and nucleotides to start transcription. SmMaf1(A) and two control proteins (GST or bovine serum albumin) did not significantly affect transcription under these conditions (Fig. 4A and data not shown). However, SmMaf1(B + C) and both of the full-length SmMaf1 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 TFIIIC-independent 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 interac-
TFIIIB does not dissociate TFIIIB/H18528 to preformed TFIIIC presence of different recombinant Maf1 proteins or a GST control protein. The complexes were resolved on a native 4% polyacrylamide gel.

TFIIIC-dependent assembly of recombinant TFIIIB subunits (rBrf1, 2 pmol; rTBP, 25 fmol; Bdp1, 200 fmol) on a tRNA gene was performed in the presence of TFIIIC and on the complexes. The assembly of TFIIIB on a tRNA gene in the presence of Maf1 and pol III (16) we next examined whether Maf1 and pol III (16) we next examined whether Maf1 could inhibit transcription from preassembled initiation complexes. The assembly of TFIIIB on a tRNA gene in the presence of TFIIIC and on the SNR6 gene in the absence of TFIIIC was allowed to proceed to equilibrium during a 1-h incubation prior to the addition of the different Maf1 proteins, pol III, and NTPs. As before, ScMaf1(A) and GST did not affect the level of transcription from either template (Fig. 4, A and C). Conversely the ScMaf1(B + C) fragment and SpMaf1 were still effective inhibitors of tRNA and SNR6 transcription, although higher amounts of these proteins were required to achieve the same level of inhibition as reactions where TFIIIB complexes were not preassembled (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 TFIIIC assembly (Fig. 4B and C), 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.

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

Next we examined whether rMafl 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 rMafl on transcription from preassembled complexes (Fig. 4, B and C) involves a post-TFIIIB recruitment step.

The first step in the assembly of TFIIIB on a tRNA gene is the recruitment of Brfl by promoter-bound TFIIIC (for a review, see Ref. 29). The ability of Mafl to block this limiting step in complex assembly was assessed by adding Brfl to mixtures containing Mafl proteins and TFIIIC-DNA. As for the assembly of TFIIIB, Mafl inhibition of Brfl recruitment onto TFIIIC-DNA complexes was complete and specific (Fig. 5C). Moreover domains B + C of Mafl, but not domain A, were functionally important in this assay in agreement with the transcription data (Fig. 4, A and B). Given that the effects of Mafl on transcription are independent of TFIIIC (Fig. 4C), the preceding results suggest that Mafl interacts directly with Brfl. This possibility was tested in pull-down assays using recombinant Brfl bound to Ni²⁺-NTA-resin. [³⁵S]Methioninelabeled S. cerevisiae and S. pombe Mafl were incubated with resin alone or Brfl-resin under stringent ionic conditions to prevent nonspecific binding. After extensive washing, the
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FIG. 6. Interactions of Maf1 with Brf1 and Pol III in control and CPZ-treated whole cell extracts. Immunoprecipitation and Western analysis were 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 immunoprecipitation (IP) lanes were probed with the precipitating antibody contained 5 and 10 μl of the eluate, respectively. Immunoprecipitation 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 A. All immunoprecipitation lanes contain 5 μl of the eluted sample.

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 Rpol2 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. Each monoclonal antibody specifically precipitated the corresponding tagged protein and reduced its level in the resulting supernatants by 85–90% (Fig. 6). 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 Rpol2-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 used here (Fig. 6, Supplemental Fig. S1B, and Ref. 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, overexpression of Maf1 (>10-fold) in yeast did not reduce the level of pre-tRNA Leu, pre-tRNA Lys, or pre-tRNA His in early to midlog phase (<2 × 10^6 cells/ml) under otherwise normal growth conditions (Supplemental 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 fmol/μg of protein) relative to Brf1 (5–10 fmol/μg of protein). These data all support a non-stoichiometric mechanism of inhibition by Maf1.

DISCUSSION

In this study, we showed 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 Ref. 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, and rapamycin (17)), we found that carbon source starvation, endoplasmic reticulum stress (5 mM DTT), and oxidative stress (0.5 mM hydrogen peroxide) conditions also require Maf1 to achieve repression of pol III transcription (Supplemental 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 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 Brf1 to minimize the differences in transcription (from 15- 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 and Ref. 17).3 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

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4 N. Desai, unpublished data.
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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 min [32]). The kinetics of repression following carbon source starvation and CPZ treatment as well as other repressing conditions is very rapid (t½ < 10 min [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 and Refs. 31 and 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 chromatin immunoprecipitation 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 preassembled 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” and Figs. 4–6). This may indicate that the activity of rMaf1 is lower 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 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 through physiologically important interactions with Brf1 and pol III but involves a non-physiologic, 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 TFIIIB-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 sumoylated (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.

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