Gene Looping Is Conferred by Activator-dependent Interaction of Transcription Initiation and Termination Machineries

Belal El Kaderi, Scott Medler, Sarita Raghunayakula, and Athar Ansari

From the Department of Biological Science, Wayne State University, Detroit, Michigan 48202

Gene looping juxtaposes the promoter and terminator regions of RNA polymerase II-transcribed genes in yeast and mammalian cells. Here we report an activator-dependent interaction of transcription initiation and termination factors during gene looping in budding yeast. Chromatin analysis revealed that MET16, INO1, and GAL1p-BUD3 are in a stable looped configuration during activated transcription. Looping was nearly abolished in the absence of transcription activators Met28, Ino2, and Gal4 of MET16, INO1, and GAL1p-BUD3 genes, respectively. The activator-independent increase in transcription was not accompanied by loop formation, thereby suggesting an essential role for activators in gene looping. The activators did not facilitate loop formation directly because they did not exhibit an interaction with the 3’ end of the genes. Instead, activators physically interacted with the general transcription factor TFIIB when the genes were activated and in a looped configuration. TFIIB cross-linked to both the promoter and the terminator regions during the transcriptionally activated state of a gene. The presence of TFIIB on the terminator was dependent on the Rna15 component of CF1 3’ end processing complex. Coimmunoprecipitation revealed a physical interaction of Rna15 with TFIIB. We propose that the activators facilitate gene looping through their interaction with TFIIB during transcriptional activation of genes.

Transcription of protein encoding genes by RNA polymerase (RNAP) involves several distinct steps including the assembly of preinitiation complex, initiation, elongation, termination, and reinitiation (1, 2). Transcription starts with the recruitment of RNAP II and the general transcription factors TFIIA, TFIIH, TFIIE, and TFIIB onto the promoter to form a preinitiation complex. RNAP II and general transcription factors are sufficient for accurate basal level transcription (2, 3). The response to activators requires additional cofactors that bring about stimulation of transcription by modifying chromatin structure in the promoter region and facilitating recruitment of RNAP II and general transcription factors to the exposed promoter (3–5). Once the gene is activated, the amount of transcripts produced is determined primarily by the number of reinitiation events (6). Despite the remarkable progress made in understanding the molecular mechanisms that govern initiation of transcription in eukaryotes, relatively little is known about the processes that mediate reinitiation.

The studies with RNAP I and III have implicated proper termination as a prerequisite for reinitiation of transcription (7, 8). During RNAP I and RNAP III-mediated transcription, termination factors help the polymerase to pause at the terminator region. This is followed by the release of the polymerase from the terminator. In RNAP I transcription, PTRF (Pol I and transcription release factor) facilitates release of the paused polymerase from the terminator region, whereas in RNAP III transcription, factor La performs an analogous function. Both PTRF and factor La have been shown to enhance reinitiation during subsequent rounds of transcription (7, 8). A termination factor-mediated transfer of yeast RNAP III from the terminator to the promoter for reinitiation has been demonstrated in vitro (9). Terminus-facilitated reinitiation was accompanied by a concomitant increase in the transcription efficiency of RNAP III. A similar termination-dependent enhancement in transcriptional productivity of human RNAPI has also been observed in a reconstituted in vitro transcription system (7). These data imply a role for termination factors in the recycling of polymerase from the terminator to the promoter to achieve higher transcription efficiency.

It was hypothesized that efficient transfer of polymerase from the terminator to the promoter is facilitated by a DNA loop between distal ends of the transcribed gene (6, 10). Although existence of such a loop has not been demonstrated during either RNAP I- or RNAP III-mediated transcription, it has been reported to form during transcription of human mitochondrial rDNA (11). Accordingly, the termination factor-mediated loop at the mitochondrial rDNA transcription unit brought about efficient translocation of RNA polymerase from the terminator to the promoter, resulting in a 15-fold stimulation of transcription. The existence of similar gene loops has been recently reported for RNAP II-transcribed genes in yeast and mammalian cells (12–16). It has been shown that RNAP II-dependent gene looping is the consequence of the physical interaction of the terminator with the promoter of the same gene and is dependent on ongoing transcription. The looping has been shown to require Ssu72 and Pta1, components of the 3’ end processing/termination machinery in yeast, and a functional 3’ poly(A) signal in mammalian cells (13, 15). The general transcription factor TFIIB, which exhibits a genetic interaction
Activator-dependent Gene Looping

with Ssu72, was also found to be essential for gene looping in yeast (17). Whether termination factors and TFII B facilitate transfer of RNAP II from the terminator to the juxtaposed promoter is not known. It is also not clear yet whether gene looping enhances transcription efficiency of protein encoding genes.

Here we demonstrate enhanced promoter-terminator interaction of several RNAP II-transcribed yeast genes during activated transcription. Gene looping was almost completely abolished in the cells lacking transcription activators. The general transcription factor TFII B contacted the UAS-bound activator and the terminator when the gene was in a looped configuration. TFII B dissociated from the terminator in the mutant of Rna15, which is a 3’ end processing/termination factor. We have earlier shown the dependence of gene looping on Ssu72 and Pta1, which are components of CPF-3’ end processing complex in yeast (13). Here we provide evidence in support of a role for the CFI complex in gene looping. We propose that transcription activators facilitate promoter-terminator association through their interaction with TFII B during induced transcription.

EXPERIMENTAL PROCEDURES

Yeast Strains—The yeast strains used in this study are listed in supplemental Table S1. Strain YMH867, which has GALI promoter upstream of BUD3 gene (GAL1p-BUD3), has been described previously (13). Strain pairs FY23 (WT) and SAM6 (met28); BY4733 (WT) and BEK3 (ino2-); and YMH867 (WT) and SAM4 (gal4-Δ) are isogenic. SAM6, BEK3, and SAM4 were constructed by replacing the entire open reading frame of Rna15, which is a 3’ end processing gene, the cells were obtained from a freshly streak plate and grown overnight in 5 ml of YP-raffinose at 30 °C with shaking. The next day, the cultures were diluted 100-fold in 200 ml of synthetic complete medium. The cells were allowed to grow at 30 °C. The samples were collected at 60, 90, and 120 min and processed accordingly for RT-PCR, CCC, or ChiP.

Capture Chromosome Conformation—The promoter-terminator interaction was analyzed by a modified version of CCC (mCCC) protocol as described by Ansari and Hampsey (13). The cells were grown as described above and cross-linked with 1% formaldehyde for 15 min at room temperature with gentle shaking. The reaction was quenched by adding glycine to a final concentration of 125 mM and incubating the culture for another 5 min at room temperature with gentle shaking. The cultures were spun down at 4 °C, and the cell pellet obtained from the 50-ml culture was washed with 10 ml of 1× TBS buffer (10 mM Tris-HCl, pH 7.5, 200 mM NaCl) containing 1% Triton X-100 followed by a second wash in 1× TBS buffer without Triton X-100 and resuspended in 400 μl of FA-lysis buffer (50 mM HEPES-KOH, pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, and 1 mM PMSF). Approximately 400 μl of acid-washed glass beads were added, and the cells were lysed by vigorous shaking for 40 min at 4 °C in a microcentrifuge tube. The microcentrifuge tube containing lysed cells along with glass beads was punctured with a 22-gauge needle, and the cell lysate was collected in a 15-ml tube following a brief spin. The filtrate was transferred into a 1.5-ml microcentrifuge tube and centrifuged for 15 min at 4 °C. The crude chromatin pellet was washed with 500 μl of FA-lysis buffer and resuspended in 500 μl of 10 mM Tris-HCl, pH 7.5.

Cross-linked, crude chromatin was digested with a restriction endonuclease (Alul) that cuts at least once in the coding region and at sites flanking the promoter and terminator regions of a gene, as shown in supplemental Fig. S1A. Typically, 80 μl of chromatin was digested with 10 μl of restriction enzyme in a 100-μl reaction volume for 4 h at 37 °C with gentle shaking. The digested chromatin was collected by spinning, and the pellet was resuspended in 90 μl of 10 mM Tris-HCl, pH 7.5. To inactivate the restriction enzyme, 10 μl of 10% SDS was added followed by incubation at 65 °C for 20 min. Only 60 μl of digested chromatin was used in the next ligation step. The ligation reaction consisted of 75 μl of 10% Triton X-100, 5 μl of Quick Ligase (New England Biolabs), and 375 μl of Quick Ligase buffer in a final volume of 750 μl. Ligation reactions were performed for 90 min at room temperature with gentle shaking. To ensure complete removal of RNA, 10 μg of DNase-free RNase was added to the reaction mixture, and the incubation was carried out for 30 min at 37 °C. The cross-links were reversed overnight at 65 °C in the presence of 100 μg of proteinase K and 1% SDS. The samples were extracted three times with phenol-chloroform and ethanol-precipitated using glycerol as a carrier. DNA concentration was determined spectrophotometrically, and 750 ng of DNA was used as the template in each PCR. The linearity of PCR was routinely determined using different amounts of template.
PCRs (30 cycles) to detect transcription-induced chromatin looping were performed using primers P1, C1, C2, C3,..,Cn and T1, as shown in supplemental Fig. S1A. Control PCR products were generated using primer pair F2, R1 corresponding to an uncut terminator proximal region of the gene tested. PCR products were fractionated on a 1.5% agarose gel and visualized by ethidium bromide staining using GEL LOGIC 200 (KODAK). Quantification was performed as described below.

The mCCC positive control PCR to check the efficiency of primer pairs was generated as described by Dekker et al. (20). The purified genomic DNA was digested with AluI and randomly ligated without dilution at a DNA concentration of 500 ng/µl.

RT-PCR—The total RNA from yeast was isolated by modification of the method described by McNeil and Smith (21). RT-PCR was carried out in two steps. In the first step, yeast poly(A) mRNA (2 µg) isolated by the protocol described above was reverse transcribed into cDNA using oligo(dT) primer and SuperScript III reverse transcriptase (200 units; Invitrogen) according to the manufacturer’s instructions. In the second step, poly(A) mRNA derived cDNA was PCR-amplified by gene-specific primers (30 cycles). Different dilutions of cDNA were used to determine the linearity of PCR.

ChIP—Cross-linking, cell lysis, and isolation of chromatin were done as described for mCCC analysis. Chromatin preparation obtained above was sheared by sonication (15 pulses of 20 s each with 1 min cooling after each pulse). Sonication was performed at the 25% duty cycle in a Branson digital sonifier. Following sonication, the samples were centrifuged at 14,000 rpm for 15 min in a refrigerated microcentrifuge. Pellet was discarded, and supernatant was used in subsequent steps.

The amount of sonicated chromatin to be used for immunoprecipitation depends on the quality of antibody and the amount of protein (antigen) present in the chromatin preparation. Approximately 5–10 µg of appropriate antibody (the amount of antibody added need to be optimized for each antibody preparation) was added to 400 µl of chromatin preparation and allowed to bind for 4 h at 4 °C with gentle shaking. The antigen-antibody complex was adsorbed onto 20 µl of protein A-Sepharose beads (beads should be prewashed with FA-lysis buffer) for 1 h with gentle shaking at 4 °C.

The beads were washed successively with 1 ml each of FA-lysis buffer containing 0.06% SDS (two times), FA-lysis buffer containing 0.06% SDS and 500 mM NaCl (two times), ChIP wash buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.06% SDS, and 1 mM EDTA) (twice) and TE buffer (once). All of the washing steps were performed at room temperature. The beads were resuspended in 250 µl of ChIP elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1% SDS), incubated at 65 °C for 10 min, and briefly spun, and the supernatant was collected and incubated with 10 µg of DNase-free RNase for 15 min at 37 °C. 20 µg of proteinase K and 2.5 µl of 10% SDS were added, and the cross-links were reversed by overnight incubation at 65 °C. The samples were extracted with phenol-chloroform at least two times followed by ethanol precipitation of DNA in the presence of glycerol as a carrier. DNA pellet was resuspended in 50 µl of water and used as template for further PCR. Primers for PCR were designed in such a way that PCR product is 200–250 base pairs in length. PCR was performed using 2–5 µl of immunoprecipitated DNA as template. PCR products were run on 1.5% agarose gel and visualized by ethidium bromide staining. Note that a negative control without antibody should always be performed.

Coimmunoprecipitation—For coimmunoprecipitation using a TAP-tagged Rna15, the cells were grown in 1 liter of YPD to an A600 of 1.5. The cell pellet obtained from the 1-liter culture was washed once with 50 ml of ice-cold 1× TBS buffer containing 1% Triton X-100 and followed by a wash with 50 ml of cold double distilled H2O. The pellet was resuspended in 9 ml of IP lysis buffer (20 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.5 mM EDTA, 1 mM MgCl2, 0.1% Triton X-100, and 1 mM PMSF) and frozen in liquid nitrogen. The frozen cell pellet was homogenized to a very fine powder using a liquid nitrogen chilled mortar and pestle, transferred to a beaker, and allowed to thaw. The resulting cell lysate was centrifuged at 16,400 rpm for 20 min in a Sorvall SS-34 rotor, and the supernatant was used for immunoprecipitation.

Cell lysate (8 ml) containing TAP-tagged protein was allowed to bind to 60 µl of IgG-Sepharose beads in a 15-ml tube. The binding was performed at 4 °C for 2 h with gentle shaking. Following binding, IgG-Sepharose beads were transferred to microcentrifuge tubes and washed three times with 1 ml each of IP wash buffer (20 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.5 mM EDTA, 1 mM MgCl2, 1 mM PMSF, and 0.1% Triton X-100). Elution was performed by resuspending beads in 100 µl of 2× Laemml buffer (25 mM Tris-HCl, 4% glycerol, 0.8% SDS, 2% β-mercaptoethanol, and 0.01% bromphenol blue) followed by incubation at 65 °C for 20 min. The eluted proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane using an Amersham Biosciences TE70 semi-dry transfer apparatus. The membrane was incubated with the first antibody (anti-TFIIB polyclonal antibody) followed by incubation with the second antibody (anti-rabbit IgG-POD conjugate). The protein bands were visualized using the Pierce Pico chemiluminescent reagents. Coimmunoprecipitation using anti-TFIIB antibodies was similarly performed except that cell lysate (8 ml) was incubated with 50 µl of TFIIB antisem, and antigen-antibody complex was allowed to bind to 30 µl of protein A-Sepharose beads. Washing and elution was done as described above, and eluted proteins were separated on 6% SDS-polyacrylamide gels. TAP-tagged Rna15 was detected by anti-calmodulin-binding protein antibodies.

For coimmunoprecipitation using HA-tagged transcription activators, the cells were grown in 1 liter of appropriate medium to an A600 of 1.0–1.2 and then induced for 2 h. Cross-linking was performed by incubating the cells in formaldehyde (final concentration, 0.5%) for 20 min, and the reaction was stopped by the addition of glycine (final concentration, 125 mM). The cell pellet was washed and lysed as described above. Cell lysate was subjected to sonication (30 pulses of 10 s each at 25% duty cycle with 30 s rest in between). The resulting cell lysate was centrifuged at 16,400 rpm for 20 min in a Sorvall SS-34 rotor. The supernatant was used for immunoprecipitation. One sample of the cell lysate, grown under induced conditions, was pre-treated with 10 units of micrococcal nuclease (Worthington...
Activator-dependent Gene Looping

Labs) for 30 min at 37 °C prior to binding. A 1-ml aliquot of the micrococcal nuclease-treated cell lysate was checked for complete digestion of chromatin by running on 1.5% agarose gel following deproteinization and decross-linking of samples.

Cell lysate (8 ml) containing HA-tagged protein was allowed to bind to 60 μl of anti-HA-Sepharose beads in a 15-ml tube. The binding was performed at 4 °C for 2 h with gentle shaking. Following binding, anti-HA-Sepharose beads were transferred to microcentrifuge tubes and washed three times with 1 ml each of IP wash buffer B (20 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.5 mM EDTA, 1 mM MgCl₂, 1 mM PMSF, and 10% glycerol). Elution was performed by resuspending beads with 100 μl of 2× Laemmlli buffer followed by incubation at 65 °C for 20 min. The eluted proteins were separated by SDS-PAGE and Western blot analysis performed with anti-TFIIB antibodies as described above.

Quantification—The gels were quantified based on the net intensity of the bands using the Kodak 2D software in conjunction with the Gel Logic imaging system. 1.5% agarose gels were stained for 30 min in 150 ml of 1× TAE with 5 μl of ethidium bromide solution (10 mg/ml). For each experiment, the global mean of the standard ladder (100 bp 50 μg/ml Quick Load; New England Biolabs, Ipswich, MA) was used to scale each gel to account for any variability in gel density and ethidium bromide staining. Using the scaled net intensities, a minimum of three trials were analyzed under the Univariate analysis of variance model in the SPSS statistical software to verify that there was no significant gel interaction ($p < 0.005$). Each trial was also duplicated to ensure that there was no significant trial interaction ($p < 0.005$). Scaled net intensities were then used to generate ratio data comparing the experimental test with that of the control PCR, which was then used to generate the mean and standard deviation as shown in the graphs. For all of the quantification graphs, the error bars represent one full unit of standard deviation based on a minimum of three trials.

RESULTS

Gene Looping Accompanies Activated Transcription—Several yeast, mammalian, and viral genes have been shown to adopt a looped configuration during transcription (12–17). We further demonstrated that the looping of galactose-inducible genes accompanied activated transcription and not the repressed or transcriptionally poised state of the gene (13). A similar activation-dependent loop formation was recently demonstrated for the human immunodeficiency virus proviral gene (15). To understand the mechanism of activation-dependent gene looping, we investigated the role of transcription activators in promoter-terminator interaction. To address the issue, we first investigated whether gene looping is unique to galactose-inducible genes or whether it accompanies transcriptional activation of other genes as well, in budding yeast. We therefore examined the architecture of three yeast genes: MET16, INO1, and GAL1p-BUD3, during their different transcriptional states. These three genes were chosen because their regulation is relatively well understood, and their transcriptional state can be regulated by simply changing the growth conditions.

Transcription of MET16 is regulated by methionine. In the presence of methionine, MET16 is transcribed at a very low level. However, upon methionine depletion, transcription of MET16 is stimulated by about 5-fold (supplemental Fig. S1B, lane 2, and Fig. 1B). Similarly, transcription of INO1, a gene involved in inositol metabolism, is enhanced by about 50-fold in the absence of inositol in the medium (supplemental Fig. S1B, lane 4, and Fig. 1C). GAL1p-BUD3, as we have shown earlier, is almost completely repressed in the presence of dextrose as a carbon source (supplemental Fig. S1B, lane 5, and Fig. 1D). In the presence of raffinose, the gene remains inactive but is in a transcriptionally poised state (supplemental Fig. S1B, lane 6, and Fig. 1D). The addition of galactose brings about a 50-fold stimulation of transcription of GAL1p-BUD3 (supplemental Fig. S1B, lane 7, and Fig. 1D).

Gene looping of MET16, INO1, and GAL1p-BUD3 was monitored by the mCCC protocol as described by Ansari and Hampsey (13). mCCC analysis was performed in the same batch of cells in which RNA analysis was done. Briefly, the cells were grown under appropriate conditions and cross-linked with formaldehyde. Cross-linked chromatin was isolated and digested with AluI, which cuts the coding regions of MET16, INO1, and GAL1p-BUD3, three, seven, and eleven times, respectively (supplemental Fig. S1A). In addition, AluI also digests upstream of the promoter and downstream of the 3′ end of all three genes. The complete digestion of genes was routinely confirmed by a diagnostic PCR using primer pair F1 and R1 as indicated in supplemental Fig. S1A (data not presented). The digestion was followed by ligation under the conditions that favor intramolecular ligation. The cross-links were reversed, and the DNA was purified by phenol-chloroform extraction and ethanol precipitation.

This purified DNA was quantified, and an equal amount was used as template in further CCC-PCRs. The interaction of promoter with the internal regions and the 3′ end of the gene was detected by PCR using primer pairs P1-C2, P1-C3, P1-C4...P1-Cn and P1-T1 as indicated in supplemental Fig. S1A. A distinct PCR product was obtained with P1-T1 primer pair for MET16 (supplemental Fig. S1C, lanes 3 and 7), INO1 (supplemental Fig. S1D, lanes 4 and 9), and GAL1p-BUD3 (supplemental Fig. S1E, lanes 5 and 11). These experiments clearly indicate that the promoter (P1) of all three genes specifically interacts with the 3′ end of the gene (T1) and not with the internal regions (C2, C3...Cn) during transcription. The P1-T1 PCR product was therefore taken as a measure of promoter-terminator interaction or gene looping in all subsequent experiments. The amount of template was adjusted to get the P1-T1 PCR product in the linear range. The ligation dependence of all PCR products was determined as described by Ansari and Hampsey (13). A control F2-R1 PCR that amplified a region near the 3′ end of the gene was routinely performed as a loading control.

The experiments described above demonstrate a physical association of the terminator with the promoter of MET16, INO1, and GAL1p-BUD3. To determine whether gene looping accompanies transcription in general or only activated transcription, mCCC analysis of MET16, INO1, and GAL1p-BUD3 was carried out under different transcriptional states of the genes. A distinct P1-T1 PCR signal was observed when transcription of MET16 was activated in the absence of methionine (Fig. 1, E, lane 2, and F). The signal decreased by about 7-fold...
when methionine was added to the medium (Fig. 1, E, lane 1, and F). This indicates that gene looping of MET16 increased during activated transcription of the gene. Similar results were obtained with INO1 and GAL1p-BUD3 genes. Gene looping as measured by the P1-T1 PCR signal was observed at INO1 and GAL1p-BUD3 loci when their transcription was activated (Fig. 1, E, lanes 6 and 11, G, and H). The looping signal for INO1 decreased by about 4-fold (Fig. 1, E, lane 5, and G) and for GAL1p-BUD3 by about 5-fold (Fig. 1, E, lane 9, and H) during repression. No significant gene looping at GAL1p-BUD3 was observed even when the gene was in a transcriptionally poised state (Fig. 1, E, lane 10, and H). To ensure that the loss of looping signal of MET16, INO1, and GAL1p-BUD3 during the transcriptionally repressed state of genes was not due to a failure of ligation step of mCCC protocol, we checked for looping of ACTIN in the same reaction mixture. A distinct P1T1 PCR signal for ACTIN was observed (Fig. 1E, lanes 15–17) under the conditions when looping of MET16, INO1, and GAL1p-BUD3 was repressed (Fig. 1E, lanes 1, 5, and 9). These experiments demonstrate that the P1-T1 looping signal of MET16, INO1, and GAL1p-BUD3 genes is significantly enhanced upon their transcriptional activation.

Gene Looping Requires Transcription Activators—We reasoned that if gene looping is playing a role in activated transcription, it will occur in an activator-dependent manner. Thus, mCCC analysis of MET16, INO1, and GAL1p-BUD3 was carried out in the absence of their transcription activators. Met28, Ino2, and Gal4 are gene-specific transcription activators of MET16, INO1, and GAL1p-BUD3, respectively (22–24).

In the absence of Met28, no activation of MET16 was observed after 1 h of depletion of methionine from the medium (Fig. 1B). Similarly, there was no activation of INO1 in the Ino2 deletion strain following 1 h of inositol depletion (Fig. 1C). The

FIGURE 1. Gene looping accompanies activated transcription. A, schematic depiction of MET16, INO1, and GAL1p-BUD3 indicating the position of Alu restriction sites (vertical lines) and PCR primers (arrows) used in mCCC analysis. B–D, quantification of transcript levels of MET16, INO1, and GAL1p-BUD3 in wild type (black bars) and activator deleted strains (gray bars) following 60 min of induction. E, mCCC analysis of MET16, INO1, and GAL1p-BUD3 in wild type and activator deleted strains following 60 min of induction. mCCC analysis of ACTIN in wild type cells represents a positive control for gene looping under uninduced conditions. The PCR products are indicated by the primers used for amplification. The F2-R1 PCR represents the loading control indicating that equal amount of template DNA was present in each of the CCC-PCRs. F–H, quantification of the mCCC results shown above in E. Dex, dextrose; Raf, raffinose; Gal, galactose; Met, methionine; Ino, inositol.
transcription of GAL1p-BUD3 also remained completely repressed in the gal4− strain following a shift to galactose containing medium for 1 h (Fig. 1D). Thus, transcription of MET16, INO1, and GAL1p-BUD3 remained unchanged after 1 h of growth under transcriptionally inducive conditions in the absence of activators. To detect gene looping, mCCC analysis was performed using the same cultures that were analyzed for transcript level. There was no increase in the P1-T1 PCR signal of MET16, INO1, and GAL1p-BUD3 in the absence of their respective transcriptional activators (Fig. 1, E, lanes 4, 8, and 14, and F–H). These data indicate that no gene loop formation occurs in the absence of transcription activators.

Activator-independent Increase in Transcription Is Not Accompanied by Gene Looping

—Met28 is the transcriptional activator of five different genes associated with the methionine biosynthetic pathway (22). In the absence of Met28, a moderate increase in transcript levels of four of these genes has been observed after prolonged growth in the absence of methionine (22). Accordingly, we observed about a 4-fold increase in the mRNA level of MET16 in the met28−/H11002 cells after 120 min of growth in the methionine-depleted medium (Fig. 2A). We also observed a similar activator-independent increase in the transcription of INO1. The mRNA level of INO1 increased by about 8-fold in the ino2− cells following a 120-min shift to inositol-deficient medium (Fig. 2B). We reasoned that if gene looping is mediated by the activator, this activator-independent increase in transcription of MET16 and INO1 will not be accompanied by a promoter-terminator interaction. Thus, mCCC analysis was performed for MET16 and INO1 in cells deleted for their activators after 120 min of growth under transcriptionally inducing conditions. An activator-independent increase in transcription was not accompanied by a corresponding increase in the P1-T1 PCR signal of MET16 and INO1.

Activator-dependent Gene Looping
Thus, gene looping does not occur during an activator-independent increase in transcription for both MET16 and INO1.

**Transcription Activators Occupy the Promoter (UAS) but Not the Terminator Region during Gene Looping**—We next asked whether activators are directly facilitating promoter-terminator interaction or whether they recruit some factor that brings about an association of the distal ends of the genes during activated transcription. We expected that if activators are directly involved in the promoter-terminator interaction, they should be contacting the distal ends of the gene upon transcriptional activation. To address the issue, ChIP was performed with anti-HA antibodies in strains harboring HA-tagged Met28, Ino2, and Gal4. ChIP analysis revealed that Met28, Ino2, and Gal4 were present on the UAS sites in both the repressed and activated transcriptional states of their respective genes (Fig. 3, B, lanes 1, 5, 9, 13, 17, 21, and 25, and C, region A). However, no signal for the activators was observed on the terminator even upon transcriptional activation (Fig. 3, B, lanes 8, 16, and 28, and C, region D). These data imply that the activators do not directly interact with the 3’ end of the gene but are facilitating gene looping by recruiting other factors that interact with both the 5’ and 3’ ends of the gene.

**TFIIB Occupies the Terminator Region during Transcriptional Activation, Whereas TBP Does Not**—TFIIB is the general transcription factor that exhibits genetic interaction with Ssu72, a component of CPF 3’ end processing complex (25). It has been recently demonstrated that TFIIB occupies both the promoter and terminator regions of PMA1 and BLM10 in an Ssu72-dependent manner (17). Whether the presence of TFIIB on terminator was dependent on transcriptional activation of PMA1 and BLM10 was not investigated. Furthermore, looping of several yeast genes was abolished in a sua7-1, a mutant of TFIIB without affecting transcription efficiency of genes (17). Because TFIIB has been previously shown to physically interact with a number of transcription activators (reviewed in Ref. 26), it was a strong candidate for the factor mediating activator-dependent gene looping. We therefore checked for the presence of TFIIB on the promoter and 3’ end regions of MET16, INO1, and GAL1p-BUD3 in different transcriptional states of the genes. As expected, TFIIB was absent from the promoter and the terminator regions of MET16, INO1, and GAL1p-BUD3.
when the genes were repressed (Fig. 4, B, lanes 1, 4, 9, 12, 17, and 20, and D, regions A and D). No signal for TFIIB was observed on the promoter or terminator, even in the transcriptionally poised state of GAL1p-BUD3 (Fig. 4, B, lanes 21 and 24, and D, regions A and D). However, TFIIB cross-linked to both the promoter and 3’ end of all three genes upon transcriptional activation (Fig. 4, B, lanes 5, 8, 13, 16, 25, and 28, and D, regions A and D). The TFIIB signal at the 3’ end in the activated state was as strong as that on the promoter. In contrast, TBP, another general transcription factor, was detected only at the promoter (Fig. 4, B, lanes 5, 13, and 25, and D, region A) and not the terminator upon induced transcription (Fig. 4, B, lanes 8, 16, and 28, and D, region D).

Because TFIIB cross-linked to the 3’ end of genes only during activated transcription, we expected TFIIB occupancy of the terminator to be activator-dependent. TFIIB ChIP was therefore repeated in the strains deleted for MET28, INO2, and GAL4. TFIIB did not cross-link to either the promoter or the terminator regions of MET16, INO1, and GAL1p-BUD3 in the absence of respective activators even under transcriptionally inductive conditions (Fig. 4C, lanes 5, 8, 13, 16, 25, and 28, and D, regions A and D). These results reiterate the role of transcription activation in TFIIB-terminator interaction and further emphasize the role of activators in loop formation.

Because activator-independent increase in transcription of MET16 and INO1 was not accompanied by gene looping (Fig. 2, C and D), we expected TFIIB to be present on the promoter but not the terminator region of MET16 and INO1 under these conditions. TFIIB ChIP was therefore performed in activator-deleted strains (SRR13 and SRR14) grown for 120 min under induced and uninduced conditions. TFIIB cross-linked only to the promoter region of MET16 and INO1 (supplemental Fig. S2, B, lane 5, and C, lane 5) but not to the terminator region (supplemental Fig. S2, B, lane 8, and C, lane 8) during activator-independent stimulation of transcription. To check that the absence of TFIIB from the 3’ end of genes was not due to an experimental defect in the ability of proteins to cross-link to the terminator region, we performed ChIP for Rna15, which is a termination factor, under identical conditions. Rna15 ChIP signal was detected on the terminator region of both MET16 and INO1 (supplemental Fig. S2, B, lane 8, and C, lane 8), thereby indicating that protein factors can be cross-linked to the 3’ end of the two genes. These results confirm that TFIIB interacts with the terminator region only when a gene is in looped configuration.

Activators Interact with TFIIB during Activated Transcription—A number of transcription activators in yeast, mammalian systems and plants have been shown to physically interact with TFIIB (26). The activator-TFIIB interaction in these studies was demonstrated either in vitro using protein affinity chromatography or in vivo by coimmunoprecipitation (reviewed in Ref. 26). In many of these studies, activator-TFIIB interaction was demonstrated in the absence of transcription. If the activators bound to their UAS site are mediating gene looping through their interactions with TFIIB, we expect activator-TFIIB interaction to occur only during activated transcription when the gene is in a looped configuration. We therefore performed coimmunoprecipitation with anti-HA antibodies in strains with HA-tagged Met28, Ino2, and Gal4. Coimmunoprecipitation was done during induced and noninduced states of MET16, INO1, and GAL1p-BUD3 in formaldehyde cross-linked cells. There was no interaction of TFIIB with Met28, Ino2, and Gal4 under noninduced conditions (Fig. 5, lanes 3, 8, and 13). TFIIB was communoprecipitated with the activators only under transcriptionally inductive conditions (Fig. 5, lanes 5, 10, and 15). Activator-TFIIB interaction was not mediated by DNA because micrococcal nuclease digestion of DNA did not abolish the interaction (Fig. 5, lanes 4, 9, and 14). The complete digestion of chromatin by micrococcal nuclease was routinely checked before performing the coimmunoprecipitation (supplemental Fig. S3). A moderate decrease in Gal4-TFIIB interaction was often observed in the absence of DNA. This suggested that Gal4 may be interacting with TFIIB under certain conditions. However, a reproducible Gal4-TFIIB interaction was always observed in the absence of DNA (Fig. 5, lane 14). These results demonstrate that activator-TFIIB interaction occurs specifically during activated transcription.

TFIIB Localization on the Terminator Requires Rna15—The association of TFIIB with the 3’ end of genes has been shown to require Ssu72, a component of the 3’ end processing CPF complex (17). Apart from CPF complex, CF1 complex is also required for cleavage/polyadenylation of mRNA in yeast (27). We therefore asked whether association of TFIIB with the terminator regions of MET16 and INO1 requires CF1 complex as well. TFIIB ChIP was repeated for MET16 and INO1 genes in isogenic wild type and rna15-2 mutant strains grown under transcriptionally inducive conditions at the permis-
TFIIB cross-linking to the terminator region, as well as transcription and gene looping of MET16 and INO1 require Rna15. A, schematic depiction of MET16 and INO1 genes indicating the positions of ChIP primer pairs. B, ChIP analysis showing cross-linking of TFIIB to MET16 and INO1. The experiment was done using isogenic strains FY23 (WT) and Rna15-2 (rna15-2). The cells were incubated at either the permissive (25 °C) or nonpermissive (37 °C) temperatures for 90 min prior to cross-linking. ChIP was performed in cross-linked cells as described under “Experimental Procedures.” C, TFIIB interacts with Rna15. Immunoprecipitations (IP) were performed with IgG-Sepharose beads in cell lysates from strain expressing TAP-tagged Rna15 (SRR1) as described under “Experimental Procedures.” SDS-PAGE and Western blot analysis were performed as described previously. A control, immunoprecipitation was performed from the strain without TAP-tagged proteins. In the reciprocal experiment, coimmunoprecipitation was performed with anti-TFIIB antibodies, and Rna15 was detected by Western blot as described previously. D and E, RNA analysis of MET16 and INO1 at permissive (25 °C) and nonpermissive (37 °C) temperatures in wild type and Rna15-2 strains. The RNA level was detected by RT-PCR as described under “Experimental Procedures.” F and G, gene looping of MET16 and INO1 detected using P1-T1 PCR signal in FY23 (WT) and RNA15-2 (rna15-2) at permissive (25 °C) and nonpermissive (37 °C) temperatures. Ab, antibody; In, input.

TFIIB Interacts with Rna15—Because Rna15 is required for the presence of TFIIB at the terminator region, we checked whether TFIIB is physically interacting with it. Coimmunoprecipitation was performed with the cell lysate containing TAP-tagged Rna15 using IgG-Sepharose beads. The presence of TFIIB in the coimmunoprecipitated fraction was detected by Western blot. TAP-tagged Rna15 was able to pull down TFIIB (Fig. 6C, lane 3). No signal for TFIIB was detected in the strains lacking TAP tag (Fig. 6C, lane 2). A reciprocal coimmunoprecipitation using anti-TFIIB antibodies followed by detection of Rna15 signal by Western blot gave identical results (Fig. 6C, lane 6). These results demonstrate a physical interaction of TFIIB with the Rna15 subunit of the CF1 complex.

Role of Rna15 in Gene Looping—Because the presence of TFIIB on the terminator was dependent on Rna15, we next asked whether it is also required for enhanced transcription and gene looping of MET16 and INO1. As expected, RNA level of MET16 and INO1 decreased by about 3- and 5-fold, respectively, following shifting of the cells to a nonpermissive temperature (37 °C) for 90 min in a rna15-2 strain (Fig. 6D and E). To determine whether Rna15 is required for promoter-terminator interaction, mCCC analysis was performed in rna15-2 strain under induced conditions at the permissive (25 °C) and nonpermissive (37 °C) temperatures in the same batch of cells where a reduction in transcription was observed. A distinct P1-T1 PCR signal was observed for MET16 and INO1 in both the wild type and rna15-2 strains at the permissive (25 °C) temperature (Fig. 6F and G). At 37 °C, the P1-T1 PCR signal was abolished in the rna15-2 strain, whereas the wild type strain showed a decrease in the P1-T1 PCR signal (Fig. 6F and G).

TFIIB is a subunit of the CF1 complex. TFIIB cross-linked to the 3’ ends of MET16 and INO1 in the wild type strains at 25 and 37 °C during induced conditions (Fig. 6B, lanes 4, 8, 20, and 24). Remarkably, however, the TFIIB cross-linking to the terminator was abolished in rna15-2 strains at nonpermissive temperature (37 °C) (Fig. 6B, lanes 16 and 32), whereas the cross-linking to the promoter remained intact (Fig. 6B, lanes 13 and 29). A corollary of these results is that TFIIB association with the CF1 complex is essential for its localization at the 3’ end of MET16 and INO1.

Activator-dependent Gene Looping—Because the presence of TFIIB on the terminator was dependent on Rna15, we next asked whether it is also required for enhanc...
signal was obtained for both MET16 andINO1 when the cells were grown at the permissive temperature (25 °C) (Fig. 6, F and G). However, upon shifting to a nonpermissive temperature (37 °C), P1-T1 looping signal decreased by about 8–10-fold for both MET16 andINO1 (Fig. 6, F and G). These results emphasize the important role of the TFIIB-Rna15 interaction during gene looping in yeast.

**DISCUSSION**

In this study, we have analyzed gene looping during transcriptional activation of protein encoding genes in budding yeast. Our results show that: (a) gene looping accompanies activated transcription and requires a transcription activator; (b) activators do not directly interact with the 3’ end of genes during activated transcription; (c) activators interact with TFIIB when a gene is activated and is in a looped configuration; (d) TFIIB cross-links to the distal ends of a gene in an activator-dependent manner; and (e) physical interaction of TFIIB with the 3’ end of genes requires Rna15, which is a termination factor. On the basis of these results, we propose a model of activator-dependent gene looping (Fig. 7). The looped configuration may facilitate termination-assisted transfer of RNAP II from the terminator to the promoter during the second and subsequent rounds of transcription. During scaffold-based reinitiation, very few components have to be recruited to the promoter to form preinitiation complex (28). The reinitiation is therefore faster than initiation (29). If RNAP II is transferred directly from the terminator to the scaffold, the rate of reinitiation is expected to be augmented even further (6). A gene looping assisted transfer of polymerase from the terminator to the promoter, with a concomitant increase in transcription efficiency, has been demonstrated during mitochondrial transcription (11). We propose that gene looping plays a similar role during RNAP II-mediated transcription as well. An activator may help to keep a gene in the activated state through multiple rounds of transcription by facilitating reinitiation through gene looping.

TFIIB plays a crucial role in gene looping. It has been proposed that the presence of TFIIB at the distal ends of a gene and a simultaneous absence of TBP from the terminator region are strong indicators of gene looping. Furthermore, we have shown that the presence of TFIIB at the distal ends of a gene increases transcription efficiency, has been demonstrated during mitochondrial transcription (11). We propose that gene looping plays a similar role during RNAP II-mediated transcription as well. An activator may help to keep a gene in the activated state through multiple rounds of transcription by facilitating reinitiation through gene looping.

**FIGURE 7. A model for activator-dependent gene looping.** Step 1, during pioneer round of transcription, activator (Act) mediates recruitment of preinitiation complex (PIC) comprised of RNAP II, TFIID, TFIIB, TFIIA, TFIIF, TFIIE, and TFIH onto the promoter. Step 2, following initiation of transcription, RNAP II is released for elongation leaving behind a subset of transcription factors (TFIID, TFIIA, TFIIE, and TFIH) that form a scaffold on the promoter. TFIIB, which dissociates from the complex following initiation, interacts with UAS-bound activator. Step 3, RNAP II, after completing elongation, enters the 3′ region of a gene where it associates with the CPF and CF1 3′ end processing/termination complexes. Following cleavage and polyadenylation, mRNA is released. TFIIB interacts with the CPF and CF1 complexes bound to the 3′ end of a gene. Simultaneous interaction of TFIIB with the promoter-bound activator and terminator-bound CPF and CF1 complexes results in the formation of a gene loop that facilitates reinitiation during second and subsequent rounds of transcription. RIC is the reinitiation complex.
role in gene looping similar to that in yeast needs further investigation.

The role of gene looping may not be restricted to activation of transcription. We expect promoter-terminator interaction to have a wider implication in eukaryotic transcription. The terminator-facilitated reinitiation may also result in an economic utilization of the limited amount of transcription factors and RNAP II molecules in the cell. It has been recently demonstrated that gene looping juxtaposes an inhibitory regulatory element located at the 3' end of BRCA1 gene near its promoter region, leading to transcriptional repression of the gene in breast tumor cell lines (16). In this case, gene looping represses rather than activates transcription. In human B- and T-lymphocytes, interaction of the promoter with the terminator region of CD68 had an effect on the splicing of its primary transcript (14). A role for gene looping in preventing transcription interference has also been proposed in budding yeast, where gene density is high with little intervening space between neighboring genes (12). Gene looping may have different consequences, but it is certainly emerging as a general, possibly ubiquitous, transcription regulatory mechanism among eukaryotes.

Acknowledgments—We are grateful to Dr. Michael Hampsey (University of Medicine and Dentistry of New Jersey), Dr. John Lopes (University of Massachusetts, Amherst), Dr. Edward Golenberg, and Dr. Victoria Meller for critical reading of the manuscript. The anti-TFIIB antibodies were a generous gift from Dr. Michael Hampsey. We are especially thankful to Dr. Carl Freeman for help with the statistical analysis of data. We thank all the members of the lab for helpful discussion.

REFERENCES
1. Hahn, S. (2004) Nat. Struct. Mol. Biol. 11, 394–403
2. Woychik, N. A., and Hampsey, M. (2002) Cell 108, 453–463
3. Roeder, R. G. (2005) FEBS Lett. 579, 909–915
4. Fishburn, J., Mohibullah, N., and Hahn, S. (2005) Mol. Cell 18, 369–378
5. Keaveney, M., and Struhl, K. (1998) Mol. Cell 1, 917–924
6. Dieci, G., and Sentenac, A. (2003) Trends Biochem. Sci. 28, 202–209
7. Jansa, P., Burek, C., Sander, E. E., and Grummt, I. (2001) Nucleic Acids Res. 29, 423–429
8. Marai, R. J., Kenan, D. J., and Keene, J. D. (1994) Mol. Cell Biol. 14, 2147–2158
9. Dieci, G., and Sentenac, A. (1996) Cell 84, 245–252
10. Kulkens, T., van der Sande, C. A., Dekker, A. F., van Heerikhuizen, H., and Planta, R. J. (1992) EMBO J. 11, 4665–4674
11. Martin, M., Cho, J., Cesare, A. J., Griffith, J. D., and Attardi, G. (2005) Cell 123, 1227–1240
12. O’Sullivan, J. M., Tan-Wong, S. M., Morillon, A., Lee, B., Coles, J., Mellor, J., and Proudfoot, N. J. (2004) Nat. Genet. 36, 1014–1018
13. Ansari, A., and Hampsey, M. (2005) Genes Dev. 19, 2969–2978
14. O’Reilly, D., and Greaves, D. R. (2007) Genomics 90, 407–415
15. Perkins, K. J., Lusie, M., Mitar, I., Giacca, M., and Proudfoot, N. J. (2008) Mol. Cell 29, 56–68
16. Tan-Wong, S. M., French, J. D., Proudfoot, N. J., and Brown, M. A. (2008) Proc. Natl. Acad. Sci. U.S.A. 105, 5160–5165
17. Singh, B. N., and Hampsey, M. (2007) Mol. Cell 27, 806–816
18. Wach, A., Brachat, A., Pohlmann, R., and Filippsen, P. (1994) Yeast 10, 1793–1808
19. Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Filippsen, P., and Pringle, J. R. (1998) Yeast 14, 953–961
20. Dekker, J., Rippe, K., Dekker, M., and Kleckner, N. (2002) Science 295, 1306–1311
21. McNell, J. B., and Smith, M. (1985) Mol. Cell. Biol. 5, 3545–3551
22. Kurash, L., Cherest, H., Surnin-Kerjan, Y., and Thomas, D. (1996) EMBO J. 15, 2519–2529
23. Ambroziak, J., and Henry, S. A. (1994) J. Biol. Chem. 269, 15344–15349
24. Fedor, M. J., and Kornberg, R. D. (1989) Mol. Cell Biol. 9, 1721–1732
25. Sun, Z. W., and Hampsey, M. (1996) Mol. Cell Biol. 16, 1557–1566
26. Deng, W., and Roberts, S. G. (2007) Chromosoma 116, 417–429
27. Mandel, C. R., Bai, Y., and Tong, L. (2008) Cell Mol. Life Sci. 65, 1099–1122
28. Yudkovsky, N., Ranish, J. A., and Hahn, S. (2000) Nature 408, 225–229
29. Jiang, Y., and Gralla, J. D. (1993) Mol. Cell Biol. 13, 4572–4577
30. Mavrich, T. N., Joshi-Kshes, I. P., Venter, B. J., Jiang, C., Tomsho, L. P., Qi, J., Schuster, S. C., Albert, I., and Pugh, B. F. (2008) Genome Res. 18, 1073–1083
31. Yochum, G. S., Rajaraman, V., Cleland, R., and McWeeney, S. (2007) BMC Mol. Biol. 8, 102