Absolute Gene Occupancies by RNA Polymerase III, TFIIIB, and TFIIIC in *Saccharomyces cerevisiae*[^1]

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A major limitation of chromatin immunoprecipitation lies in the challenge of measuring the immunoprecipitation effectiveness of different proteins and antibodies and the resultant inability to compare the occupancies of different DNA-binding proteins. Here we present the implementation of a quantitative chromatin immunoprecipitation assay in the RNA polymerase III (pol III) system that allowed us to measure the absolute in vivo occupancy of pol III and its two transcription factors, TFIIIC and TFIIIB, on a subset of pol III genes. The crucial point of our analysis was devising a method that allows the accurate determination of the immunoprecipitation efficiency for each protein. We achieved this by spiking every immunoprecipitation reaction with the formaldehyde cross-linked in vitro counterparts of TFIIIB-, TFIIIC-, and pol III-DNA complexes, measuring the in vitro occupancies of the corresponding factors on a DNA probe and determining probe recovery by quantitative PCR. Analysis of nine pol III-transcribed genes with diverse sequence characteristics showed a very high occupancy by TFIIIB and pol III (pol III occupancy being generally ~70% of TFIIIB occupancy) and a TFIIIC occupancy that ranged between ~5 and 25%. Current data suggest that TFIIIC is released during transcription in vitro, and it has been proposed that TFIIIB suffices for pol III recruitment in vivo. Our findings point to the transient nature of the TFIIIC-DNA interaction in vivo, with no significant counter-correlation between pol III and TFIIIC occupancy and instead to a dependence of TFIIIB-DNA and TFIIIC-DNA complex maintenance in vivo on pol III function.

The transcriptome of RNA polymerase III (pol III)[^3] in haploid *Saccharomyces cerevisiae* consists of 274 tRNA genes, 100–200 tandem copies of *RDN5* (encoding 5 S rRNA), *SNR6* (encoding the spliceosomal U6 snRNA), *RPR1* (encoding the RNA component of RNase P), *SCR1* (encoding 7SL RNA of the signal recognition particle), *SNR52* (encoding a snoRNA involved in 2′-O-methylation of rRNA), and *RNA170* (function unknown) (recently reviewed in Ref. 1). Transcription of the tRNA genes accounts for 10–15% of nucleoside triphosphates consumed by nuclear transcription (2); reflecting this high energetic cost, pol III transcription is regulated in response to environmental stress and nutrient availability through multiple signaling pathways that converge on Maf1, the central repressor of pol III transcription (3). The pol III transcription apparatus of *S. cerevisiae* consists of the 17-subunit polymerase, the monomeric 5 S rRNA transcription-specific TFIIIA, the six-subunit TFIIIC with its two subdomains τA (subunits Tfc1, Tfc4, and Tfc7) and τB (subunits Tfc3, Tfc6, and Tfc8), and the three subunits of TFIIIB (Brf1, Bdp1, and the TATA-binding protein, TBP). The genes encoding pol III, TFIIIA, TFIIIC, and TFIIIB are all essential for yeast viability (4, 5), and each subunit has a counterpart in humans (6) with the addition of a Brf1 parologue, Brf2, that replaces Brf1 for transcription of vertebrate class 3 genes containing external promoters (7, 8). A growing body of evidence also indicates that vertebrate Maf1 is a major negative regulator of pol III transcription and that, like its *S. cerevisiae* counterpart, it is hypophosphorylated under repressive (serum-starved) growth conditions, is associated with pol III-transcribed genes, and interacts with both Brf1 (and Brf2) and the largest subunit of pol III (9–12).

TFIIIC is required for transcription of all pol III-specific genes in *S. cerevisiae*. With the exception of *RDN5* (where TFIIIA serves as a platform for TFIIIC binding), TFIIIC binds through its flexibly linked τA and τB domains to two promoter elements, boxA and boxB, that are variably separated by ~20–200 bp (but generally 30–60 bp) of intervening sequence. With one exception, these promoter elements lie within pol III-transcribed sequence (the exception being *SNR6*, whose boxB lies 120 bp downstream of the transcriptional terminator). Intraglobally bound TFIIIC serves two roles: first, it assembles TFIIIB at a site centered ~25 bp upstream of the start site of transcription through interaction between the Tfc4 subunit of τA and the Brf1 subunit of TFIIIB (reviewed in Ref. 13); second, it prevents the encroachment of repressive chromatin (14, 15). The TFIIIB-DNA complex suffices for pol III recruitment during rapidly iterating rounds of transcription in vitro (16); the low levels of enrichment of TFIIIC subunits relative to TFIIIB subunits in chromatin immunoprecipitation (ChIP) analysis (17–19) may indicate that, once bound to DNA, TFIIIB also suffices for pol III recruitment in vivo (17).

[^1]: The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3 and Figs. S1 and S2.

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[^4]: The abbreviations used are: pol, RNA polymerase; Tf, transcription factor; ChIP, chromatin immunoprecipitation; qPCR, quantitative PCR; EMSA, electrophoretic mobility shift analysis; IP, immunoprecipitation.
TFIIB can bind independently of TFIIC to TATA boxes that are present upstream of SNR6 and a few tRNA genes in vitro (20, 21), but its assembly onto DNA is entirely dependent on TFIIC in vivo and in the context of chromatin in vitro (22, 23). Although the TFIIB-binding site upstream of pol III-transcribed genes is generally AT-rich (24), TFIIC can assemble TFIIB onto an entirely GC-containing sequence (albeit less efficiently) with the resulting TFIIB-DNA complex maintaining the unique property that characterizes complexes with natural tRNA genes and with the TATA box-containing SNR6: resistance to dissociation by high salt concentrations and by polyanyins such as heparin (25). In fact, no dissociation of a fully recombinant TFIIB-DNA complex is observed after 2 h in the presence of 200 μg/ml heparin. This great stability of the TFIIB-DNA complex suggests that its occupancy on pol III genes should be high in the cell. Although Ma1 inhibits the de novo assembly of TFIIB onto DNA (26), it has little or no effect on preformed TFIIB complexes in vivo (17). The first definitive analysis of occupancy of the pol III transcription apparatus was performed at the 100–200 copies of RDN5 in vivo footprinting (27); occupancy by TFIIB was determined to be between 23 and 47%, and pol III occupancy in open promoter complexes was seen to be between 8 and 17%. An elegant quantitative electron microscopic analysis of pol III bound to the RDN5 genes (published while this manuscript was in review) is consistent with the above footprinting analysis and indicates that 20–30% of the RDN5 gene copies are actively transcribed (28).

We have been interested in how properties of pol III transcription complexes that have been observed in vitro are manifested in terms of absolute gene occupancies in vivo and have approached this problem with a modified ChiP analysis. ChiP is a powerful methodology for enumerating the components of chromatin, determining their disposition on the genome, and specifying correlations between changes of disposition and composition on the one hand with changes of the functional state of genes on the other. ChiP measurements are currently made on populations of cells, and various statistics-based analytical schemes are available for assessing the enrichment of individual DNA segments in the immunoprecipitate (IP) relative to some selected standard (generally one or more genomic regions where the protein of interest is not expected to bind). Enrichment is the product of two quantities: IP efficiency and occupancy. IP efficiency is antibody-specific; differences of IP efficiency may be encountered when different proteins are tagged with the same epitope, and even with different placements of an epitope tag on a protein (essentially because partners or neighbors of the tagged protein can expose or mask epitopes to varying extent). Thus, deconvoluting IP enrichment to specify site occupancy is an unsolved problem. Although it stands to reason that there must be a general correlation between IP enrichment and gene occupancy, it would be helpful to be able to convert shared belief to experimental quantification.

In the work that is presented, we have spiked formaldehyde-treated cell lysates with in vitro assembled and formaldehyde-cross-linked pol III transcription complexes as a means of determining chromatin immunoprecipitation efficiencies to derive protein occupancies of a diverse group of pol III-transcribed genes. Occupancy by TFIIB of all but one transcriptionally functional pol III gene was near saturation levels (>80%) with pol III occupancies on average 60% of TFIIB occupancy. In contrast, TFIIC occupancies on average were only 14% of TFIIB occupancies, indicating that TFIIC is not directly involved in pol III recruitment in vivo. In an attempt to observe a counter-correlation between TFIIC occupancy and ongoing transcription by pol III, we introduced a plasmid vector containing a modified SUP4 tRNA gene that allows TFIIB and TFIIC to form their promoter complex but greatly reduces the ability of pol III to initiate transcription. Surprisingly, the TFIIC and TFIIB occupancies of this gene were greatly diminished and, moreover, to an even greater extent than pol III occupancy. The possible implications of this finding are also discussed.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Plasmids**—Thirteen copies of the c-Myc epitope were inserted at the C termini of RET1, BDPI, TFC4, TFC1, TFC6, TFC7, and TFC8 in yeast strain BY4727 (ATCC) by homologous recombination of a 13Myc-KanMX PCR product (29) generated with primers specified in supplemental Table S2. TA30pyr was obtained by PCR amplification of plasmid pCJ-TA30 (25) with primer 5′-TATAAGCCGCGTCTCCTACTCTTTCTCTTGTCTTCTTCGTAAGCC-3′ and a T7 promoter primer and cloned into the SacII and EcoRI sites of pCJ-TA30. TA30 and TA30pyr were excised as BamHI fragments and inserted into the BamHI site of pRS316. Plasmid pRS316-TA30-α70-tQ-tl and its pyr variant were constructed as follows: tl(CAA)DR2 was amplified by PCR from genomic DNA (bp −76 to +205, relative to the start site of transcription as +1) and inserted into the SmaI site of vector pGEMI. tl(CAA)DR2 was excised as an Xbal-EcoRI fragment, and tQ(UGG)L was excised as an Xbal-HindIII fragment from plasmid pPC6 (30), and both fragments were simultaneously ligated between the EcoRI and HindIII sites of pET21b-RpoD(1–571) (31). The two tRNA genes and the rpoD segment were excised with SacII (filled in) and NotI and ligated into the NotI and XbaI segment were excised with SacII and NotI and ligated into the NotI and XbaI.

**ChiP and Quantitative PCR (qPCR)**—Yeast cultures (100 ml) were grown at 30°C in YPD to A500 of ~0.8. Formaldehyde cross-linking and ChiP followed procedure and buffers specified by Strahl-Bolsinger et al. (32), after scaling with respect to culture volume and with the following modifications. Cross-linked and washed cells were resuspended in 600 μl of lysis buffer (50 mM Na-Hepes, pH 7.8, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl chloride, 1 mM benzamidine, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin) and broken with glass beads for 30 min at 4°C. The whole lysate was sonicated to an average DNA fragment size of ~500 bp. Sonicated chromatin, corresponding to 600 μg of total protein/ChIP sample, was preincubated with 40 μl of protein G-agarose for 1 h at 4°C, and the supernatant fluid was recovered. In vitro assembled and cross-linked protein-DNA complex was added to this material (see under “EMSA” below).

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*4 D. F. Steiner and G. A. Kassavetis, unpublished observations.*
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An aliquot was then saved as total input DNA/protein reference, and the remainder was incubated with 10 μg of mouse monoclonal anti-Myc antibody overnight at 4 °C. Protein G-agarose beads (40 μl) were added for 2 h at 4 °C. The beads were recovered and washed twice as cited above, and cross-linked protein-DNA complexes were eluted with 2 × 75 μl of elution buffer (50 mM Tris-HCl, pH 8, 1% SDS, 10 mM EDTA) for 10 min at 65 °C. Cross-linking was reversed by incubation for 6 h at 65 °C. An aliquot of this material was saved for Western blot analysis (see below), and the DNA of the remainder was purified on a PCR purification spin column.

qPCR was performed by titrating total input DNA and ChIP DNA in the presence of [α-32P]dCTP, using the primers listed in supplemental Table S3 (M13 forward and reverse primers were used for amplification of TA30 and TA30pyr in pRS316) and quantified by PAGE and phosphorimaging plate analysis. The spiked complex ChIP recovery was quantified by scintillation counting and by qPCR (with SP6 and T7 promoter primers). Fractional occupancy for each pol III-transcribed gene was determined as (N - C)/(S - C), where N, S, and C are the fractions of input DNA that were immunoprecipitated for the gene of interest (N), for the added spiked complex (S), and for the Tfc4 open reading frame-negative control (C), respectively.

Western Blotting—Immunoprecipitation efficiency by Western blot analysis (33) was based on titrations of total input and immunoprecipitated and eluted proteins (after reversal of cross-linking). Anti-Myc antibody was detected with rabbit anti-mouse IgG and 125I-protein A and quantified by phosphorimaging plate analysis.

EMSA—The Brα fraction from 80 g of yeast expressing either Bdp1-Myc, Tfc4-Myc, Tfc6-Myc, or C128-Myc was prepared as described (34). The Brα fraction containing Tfc4/6-Myc or C128-Myc was dialyzed and loaded onto DEAE-Sephadex followed by elution with buffer containing 250 mM NaCl or 500 mM NaCl, respectively, as previously described (30). An internally 32P-labeled probe was prepared by PCR amplification of the modified SLIP4 tRNA gene TA30 (25) with primers 5'-AGCGAATTCATTTAGTGACACTAGATAAGGTTCTCAGCTCATTAGGGAGCGTCTGAGAATGG-3' and 5'-AGCGAATTCATTTAGTGACACTAGATAAGGTTCTCAGCTCATTAGGGAGCGTCTGAGAATGG-3' (thus adding SP6 and T7 promoter sequences to the ends) and Pfu DNA polymerase and purified by nondenaturing PAGE. Protein-DNA complexes were formed with 2 fmol of probe at 20 °C for 40 min in 40 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.1 mg/ml bovine serum albumin, and 0.1 mg/ml PUC19 or pLNG56 (30) (the latter containing the SLIP4 gene G56 promoter-down TFIIIC binding-defective mutation).

For protein-DNA complex formation with Myc-tagged TFIIIC subunits, reactions were supplemented with 100 fmol each of recombinant Brf1, TBP, and Bdp1 (rTFIIIB) (35) and incubated for 40 min at room temperature. Formaldehyde was added to 0.5% (v/v) for 5 min, and cross-linking was terminated by the addition of glycine to 0.36 M. After challenge with 0.2 mg/ml heparin for 2 min, the samples were loaded for electrophoresis on a 4% polyacrylamide-TE gel (34) to resolve and quantify the heparin-resistant TFIIIC-TFIIIB-DNA complex. For in vitro assembled Myc-tagged TFIIIB-containing complexes, the Brα fraction from a BDP1-Myc cell extract was incubated with probe DNA, 100 fmol of recombinant TBP, and 100 fmol of recombinant Brf1. After a 40-min incubation at room temperature, formaldehyde was added to 0.5% (v/v) for 5 min, and cross-linking was terminated with glycine added to 0.36 M. After challenge with 0.2 mg/ml heparin for 2 min, the samples were subjected to electrophoresis as above to resolve and quantify the heparin-resistant TFIIIC-TFIIIB-DNA and TFIIIB-DNA complexes.

RESULTS

The genes encoding the second largest subunit of pol III, C128, the Bdp1 subunit of TFIIIB, and the Tfc4 subunit of TFIIIC were modified to contain 13 copies of the c-Myc epitope tag at their C termini (29) in individual S. cerevisiae strains. Two methods for quantifying the efficiency of chromatin immunoprecipitation were compared. In the first method, Western analysis with anti-Myc antibody and 125I-protein A was used to compare the efficiency of immunoprecipitation of each tagged subunit from their respective formaldehyde-treated cell lysates. This simple approach does not take into account differences in the precipitation efficiency of TFIIIB, TFIIIC, and pol III bound to DNA in the context of cross-linked chromatin or in their respective unbound (free) states. A second method was devised to directly measure the immunoprecipitation efficiency of formaldehyde-cross-linked TFIIIB-, TFIIIC-, and pol III-DNA complexes with the aid of their in vitro assembled counterparts. Partially purified fractions containing Bdp1-Myc, Tfc4-Myc, or
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When formaldehyde-treated complexes containing Myc-tagged Tfc4, Bdp1, or C128 were produced, a small portion was retained for EMSA (Fig. 1, B–D); the remainder was used to spike the corresponding formaldehyde-treated cell lysate. In each case, we endeavored to cross-link and quantify higher order complexes that would more closely reflect those that would assemble in vivo. For lane 3 of Fig. 1B, TFIIB-TFIIC-DNA complexes were formed with a DEAE Sephadex fraction containing Tfc4-Myc-tagged TFIIC supplemented with recombinant TFIIB and reacted with formaldehyde before stripping with heparin. The TFIIC-DNA and TFIIB–TFIIC-DNA complexes and the smear above the latter complex (containing, in addition, pol III and/or nonspecifically binding proteins) were quantified together as containing TFIIC. Here, the presence of the TFIIB-DNA complex is due to TFIIC release by formaldehyde, which occurs to variable extent, rather than to heparin stripping of un-cross-linked TFIIC (data not shown). For formation of Bdp1-Myc-tagged TFIIB-TFIIC-DNA complexes, the corresponding Brα fraction was supplemented with recombinant Brf1 and TBP. Radioactivity in the formaldehyde-treated TFIIB-TFIIC-DNA and TFIIB-DNA complexes (Fig. 1C, lane 2) was combined for quantifying Bdp1-Myc (the sum closely matching radioactivity in the TFIIB-DNA complex released by heparin in the absence of cross-linking; lane 1).

Although it is possible to resolve the pol III-TFIIB-TFIIC-DNA and TFIIB-TFIIC-DNA complexes electrophoretically, release of TFIIB by formaldehyde would generate a pol III-TFIIB-DNA complex that is not resolved from the TFIIB-TFIIC-DNA complex. Accordingly, to be able to quantify formaldehyde cross-linked complexes containing Myc(C128)-tagged pol III, we took advantage of the fact that the transcript-elongating complex formed on the SUP4 gene in the absence of GTP (containing a 17-nt nascent transcript) is resistant to dissociation by heparin, as is TFIIB (Fig. 1D, lane 2) (30); in contrast, heparin strips pre-elongating pol III as well as TFIIC (lane 1). pol III-TFIIB-TFIIC-DNA complexes were formed with recombinant TFIIB and the DEAE Sephadex fraction containing C128-Myc pol III and TFIIC; ATP, CTP, and UTP were added; TFIIC and uninitiated pol III were stripped with heparin prior to formaldehyde treatment. The resulting pol III-TFIIB-DNA complex was quantified (lanes 3–5).

Determinations of the ChIP efficiency of in vitro assembled complexes introduced into formaldehyde-treated cell lysates on the basis of recovery of 32P-labeled probe and qPCR were in good agreement (32P/PCR = 1.15 ± 0.24). qPCR-derived values of ChIP efficiency were used for all of the analyses that follow.
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We next compared the immunoprecipitation efficiency of total Myc-tagged subunit by quantitative Western blotting with that of the spiked complex in the same cell lysates (Table 1). Whereas the IP efficiencies of total Tfc4-Myc and Tfc4-Myc present in a TFIIIB-TFIIIC-DNA complex were comparable, the IP efficiencies of DNA-bound Bdp1-Myc and C128-Myc were 2.0- and 2.7-fold higher, respectively. Although the in vitro cross-linked complexes do not perfectly mimic complexes assembled and cross-linked in vivo, gene occupancy values for TFIIIB and pol III determined on the basis of spiked complex immunoprecipitation efficiency were in line with expectation, whereas occupancy values based on quantitative Westerns far exceeded 100%.

The spiked-complex procedure was used to quantify TFIIIC, TFIIIB, and pol III occupancy of genes with diverse sequence characteristics that might influence occupancy (Table 2). For example, tRNA genes *tP(AAG)CR* and *tT(TGT)HR2* have initially transcribed sequences that may diminish transcription and pol III occupancy; consequently, if active transcription across boxB releases TFIIIC, these genes might display increased levels of TFIIIC occupancy. Likewise, the upstream DNA sequence contexts of tRNA genes *tH(TAT)DR2* and *tW(CCA)GL* might enhance and decrease TFIIIB binding, respectively. Two anomalous loci were included because they have been determined (by qPCR) to contain TFIIIC with diminished levels of (ZOD1) or no (ETC8) TFIIIB and pol III. The outcome of this analysis is summarized in Table 3.

TFIIIB occupancy of the five tRNA genes was extremely high (~80–120%), as was occupancy at SCR1, encoding the 7SL RNA of the signal recognition particle (~90%; Table 3). Even taking into account the margin of error and the above-noted underestimation of the spiked complex IP efficiency by PCR, these values suggest some maintenance of TFIIIB-DNA complexes within the 15–20% of cells undergoing mitosis (38). The occupancy of TFIIIB at the U6 snRNA gene, SNR6, was ~50%. It is noteworthy that pol III occupancy of these genes (excluding the anomalous ZOD1 and ETC8 loci) was generally ~70% of TFIIIB occupancy. We also note that the two genes with the lowest relative pol III occupancy, *tP(AAG)CR* and *tT(TGT)HR2* (~40%), were selected precisely for that potentiality (Table 2). TFIIIC occupancy ranged between ~5 and 25% on all genes (including the ZOD1 and ETC8 loci). TFIIIC occupancy relative to that of TFIIIB correlated well (~15%, again excluding the ZOD1 and ETC8 loci). The higher relative TFIIIC abundance at *tT(TGT)HR2* (~25%) stands out and may be a consequence of the lower relative occupancy of pol III on this gene, but the similar level of pol III relative to TFIIIB at *tP(AAG)CR* did not correlate with a high occupancy by TFIIIC relative to TFIIIB. The SCR1 gene was partly chosen on the basis of its highly divergent boxB TFIIIC-binding site (15), which includes an A54 mutation that lowers the affinity for TFIIIC more than 40-fold (36). Consistent with the suboptimal SCR1 boxB, the occupancy of TFIIIC relative to TFIIIB was ~2-fold lower than for the other genes of the set, with the exception of *tW(CCA)GL*, which was also ~2-fold lower. Occupancy of TFIIIC, TFIIIB, and pol III at the anomalous loci ZOD1 (zone of disparity 1) and ETC8 (extra TFIIIC 8) correlated well with relative occupancy previously determined by qPCR (18); TFIIIC occupancy at ETC8 was at the low end of the range.

### TABLE 1

| Subunit       | EMSA/Western* |
|---------------|---------------|
| Tfc4-Myc      | 1.1 ± 0.1     |
| Bdp1-Myc      | 2.0 ± 0.4     |
| C128-Myc      | 2.7 ± 0.3     |

* Averages of three independent chromatin immunoprecipitations with S.E. shown.

### TABLE 2

Genes analyzed for TFIIIC, TFIIIB, and pol III occupancy

| Gene          | Features                                                                 |
|---------------|---------------------------------------------------------------------------|
| *tH(AAT)BL*   | Typical intron-less tRNA gene                                             |
| *tH(TAT)DR2*  | 60-bp intron; 94-bp suboptimal boxA-boxB separation; upstream TATA box   |
| *tP(AAG)CR*   | Lacks YR motif at consensus initiation position; functions poorly for transcription in vitro (24) |
| *tW(CCA)GL*   | GC-rich upstream TFIIIB-binding site                                       |
| *tT(TGT)HR2*  | T5 termination site within initially transcribed sequence                 |
| SNR6          | boxB lies downstream of transcription unit; suboptimal boxA-boxB separation; upstream TATA box |
| SCR1          | Longest (522 bp) pol III transcription unit; upstream TATA box; deviant boxA and boxB |
| ZOD1          | Relatively high TFIIIC ChIP enrichment but low TFIIIB and pol III ChIP enrichment (18) |
| ETC8          | Only TFIIIC occupancy (18)                                               |
| TFC4          | pol II-transcribed ORF (negative control)                                 |

### TABLE 3

Percentage of occupancy by TFIIIC, TFIIIB, and pol III

| Gene          | TFIIIC (Tfc4)* | TFIIIB (Bdp1)* | pol III (C128)* | TFIIIC/TFIIIB | pol III/TFIIIB |
|---------------|---------------|---------------|----------------|---------------|----------------|
| *tH(AAT)BL*   | 17 ± 0.5      | 123 ± 25      | 94 ± 4         | 0.14          | 0.76           |
| *tH(TAT)DR2*  | 11 ± 1.2      | 79 ± 18       | 56 ± 11        | 0.14          | 0.71           |
| *tP(AAG)CR*   | 13 ± 0.3      | 112 ± 26      | 42 ± 5         | 0.12          | 0.37           |
| *tW(CCA)GL*   | 6 ± 0.7       | 98 ± 25       | 50 ± 10        | 0.06          | 0.51           |
| *tT(TGT)HR2*  | 26 ± 3.3      | 90 ± 13       | 38 ± 10        | 0.29          | 0.43           |
| SNR6          | 7 ± 0.9       | 46 ± 7        | 31 ± 5         | 0.14          | 0.67           |
| SCR1*         | 6 ± 0.7       | 87 ± 16       | 59 ± 8         | 0.07          | 0.68           |
| ZOD1*         | 24 ± 0.8      | 35 ± 6        | 6 ± 1.1        | 0.68          | 0.18           |
| ETC8          | 5 ± 0.7       | 0.2 ± 0.1     | 0 ± 0.0        | 0.34          | 0.27           |
| TFC4          | 1 ± 0.1       | 0.7 ± 0.05    | 0.4 ± 0.06     | 0.27          | 0.27           |

* Averages of three independent chromatin immunoprecipitations with S.E. shown.

* Promoter-proximal primer set 1.
for the other eight genes, with negligible levels of TFIIIB and pol III, and TFIIIC occupancy at ZOD1 was at the high end of that range, with TFIIIB occupancy 2–3-fold lower and pol III 5–10-fold lower relative to the other genes.

SNR6 was chosen for analysis partly for the placement of its high affinity TFIIIC-binding boxB site ~120 bp downstream of the transcriptional terminator. If displacement of TFIIIC by pol III transcription through the intragenic boxB element on all other pol III-transcribed genes is responsible for the observed low TFIIIC/TFIIIB occupancy ratios, one might expect to see a higher relative TFIIIC (Tfc4-Myc) occupancy of this gene. This was not the case (Table 3). We considered the possibility that because Tfc4-Myc is part of the τA subcomplex that binds to the start site-proximal boxA promoter element, formaldehyde treatment may fail to covalently link the τA subcomplex to DNA indirectly through the boxB-binding τB subcomplex. To exclude this eventuality, we repeated the spiked complex occupancy analysis with a yeast strain harboring Tfc6-Myc. Tfc6 (τB) is part of the τB subcomplex and cross-links to DNA at the start site-distal side of boxB (39). No significant difference was observed between TFIIIC occupancy based on Tfc6 (τB) and Tfc4 (τA) cross-linking at SNR6 and three other loci (Fig. 2).

Because the above attempts to assess the existence of a counter-correlation between pol III and TFIIIC occupancy were inconclusive, we constructed a SUP4 tRNA gene that compromises transcriptional initiation by eliminating purine residues on the nontranscribed strand within 10 bp of the normal start site (TA30ppy; Fig. 3A). This altered gene and its reference type (TA30) were separately inserted into the centromeric vector pRS316 and transformed into the yeast strains expressing Myc-tagged Tfc4, Bdp1, and C128. A mutant SUP4 tRNA gene lacking a purine within 7 bp of the normal start site for transcription functions poorly in vitro and fails to suppress ochre mutations in vivo (40). The ability of TA30ppy to function for transcription was compared with its parental TA30 construct by primer extension of total yeast RNA with reverse transcriptase using a SUP4 intron-complementary primer with the ochre-suppressing mutation at its 3’ end. Because pre-tRNA transcripts are processed rapidly, the relative abundance of primary transcripts measured by primer extension with intron-specific primers is an approximate measure of the relative rate of transcription. Lane 1 of Fig. 3B shows a sample primer extension analysis of yeast harboring the reference SUP4 gene on pRS316-TA30. The +13 5’ end results from 5’-end processing, which generally precedes intron removal, because splicing of unprocessed tRNAs occurs at a substantially slower rate (41). Cells harboring pRS316-TA30ppy yielded substantially reduced levels of primer extension products (Fig. 3B, lane 2) with 5’ ends corresponding to initiation at bp +1 and +2. Surprisingly, a 5’ end mapping to +18 was also detected with this template, but not the reference SUP4 gene.

Whether this 5’ end is generated by a new initiation site or by aberrant processing was not established, but the +1, +2, and +18 primer extension products for SUP4-TA30ppy were collectively compared with +1 and +13 primer extension products for SUP4-TA30 in estimating that the relative rate of transcription of the TA30ppy gene was at least 6-fold lower than its TA30 reference counterpart. This is an overestimation of the residual activity of TA30ppy because a low level background of +1 and +2 5’ ends generated by RNA from cells lacking the pRS316 plasmids was not subtracted. (Presumably this background is due to pre-tRNA Tyr that contains the identical intron but lacks the ochre suppressor mutation.) The spiked complex procedure was used to quantify occupancy of these plasmid-borne...
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SUP4 genes (left half of Fig. 3C). TFIIIB occupancy of the SUP4-TA30 gene was at least 2-fold lower relative to the other analyzed tRNA genes (Table 3). This may be a consequence of the exceptionally GC-rich upstream DNA sequence context of SUP4-TA30 with only a partial TATA box for nucleation of TFIIIB-DNA complex formation (Fig. 3A). The occupancy of TFIIIC and pol III relative to TFIIIB (0.23 and 0.37, respectively) was within the range observed for other tRNA genes. Strikingly, the effect of the ppy variation on occupancy was the opposite of what had been anticipated: TFIIIC occupancy was reduced ~10-fold (to less than 1%) and TFIIIB occupancy was reduced ~6-fold, whereas pol III occupancy was reduced only ~3-fold.

Because the dispersed chromosomal tRNA genes cluster and co-localize in the nucleolus or at its periphery (42), we considered the possibility that the greatly reduced occupancy of the TA30pyr gene by all components of the pol III transcription apparatus could partly reflect a peculiarity of localization of a plasmid containing only a single tRNA gene. To assess this possibility, two additional tRNA genes, tQ(UGG)L and tL(CAA)C, were inserted into each plasmid, separated from SUP4-TA30 by a ~1600-bp spacer to prevent co-immunoprecipitation of SUP4 with complexes assembled on the other two tRNA genes. The presence of the additional tRNA genes did not mitigate the deficiency of TA30pyr transcription complexes (right half of Fig. 3B). The implications of the loss of TFIIIC and TFIIIB because of an 8-bp change in the initially transcribed segment of SUP4 are discussed below.

pol III binds to the preformed TFIIIB-promoter complex and executes the initial round of transcription slowly relative to reinitiated subsequent rounds of transcription. This process of rapid reinitiation, termed facilitated recycling, occurs without release of pol III from the transcription unit, suggesting that pol III is directly handed off from the terminator back to the TFIIIB-DNA complex. TFIIIB suffices for facilitated recycling on short transcription units, but TFIIIC is additionally required on longer units, such as the 520-bp SCR1 (43). Curiously, a prior qPCR analysis with tiled amplicons indicated relatively uniform TFIIIC occupancy throughout the SCR1 gene in exponentially growing cells (19) and TFIIIB occupancy of the SCR1 terminator region at 20–25% of TATA box occupancy.

These results encouraged us to apply quantitative ChIP at SCR1 using an overlapping set of PCR amplicons (Fig. 4A). TFIIIB and TFIIIC occupancy dropped off significantly within the middle of the gene (Fig. 4B: 20 and 30%, respectively, for probe 3 relative to probe 1) and was quite low at the terminator (less than 5% occupancy for probe 5 relative to probe 1). In contrast, pol III occupancy was maintained at relatively high levels throughout the SCR1 gene but decreased progressively with increasing distance from the start site (~60% occupancy with probe 5 relative to probe 1). The diminution of pol III occupancy in the downstream segment of the transcription unit is consistent with termination and pol III release not representing a rate-limiting step for transcription in the cell. The substantial decrease of TFIIIC and TFIIIB occupancy with probe 4 in respect to probe 1, and the absolute levels of pol III with both probes 1 and 4 suggest more than one pol III elongation complex simultaneously traversing the SCR1 gene. This would contribute to the high abundance of 7SL RNA in yeast (0.2% of total RNA (44), or approximately one signal recognition complex for every forty 80 S ribosomes on the basis of their respective cellular RNA content). French et al. (28) have also estimated that SCR1 would need to be simultaneously transcribed by multiple elongation complexes to generate the required abundance of 7SL RNA.

DISCUSSION

We have pursued the quantitative determination of (absolute) occupancy by TFIIIC, TFIIIB, and pol III on a sequence-distinctive group of pol III-transcribed genes for its intrinsic interest and also because our quantification could be used as a standard for interpreting qPCR-derived measurements of ChIP enrichment in terms of site occupancy.

The validity of our approach is contingent on the suitability of in vitro assembled complexes for standardization. The in vitro cross-linked complex is admittedly formed in an environment that does not match cellular chromatin. Although we have deliberately used crude yeast fractions for complex formation, it is necessary to add carrier DNA to these extracts to sequester nonspecifically DNA-binding proteins so that TFIIIC-, TFIIIB-, and pol III-DNA complexes can be identified and quantified. On the other hand, it is unlikely that the additional proteins present in cross-linked yeast chromatin substantially mask the Myc epitope of Bdp1 and C128, because this would imply true occupancies greater than 100% (Fig. 4 and Table 3). We conclude that it is also highly unlikely that the Myc epitope of Tfc4 is differentially masked in the context of yeast chromatin, because one would expect such masking to vary between Myc tags placed on different TFIIIC subunits. On the contrary, no significant difference of TFIIIC occupancy was found for TFIIIC Myc-tagged on the Tfc4 and Tfc6 subunits (Fig. 2). Estimates of TFIIIC occupancy at two tRNA genes based on Western-derived IP efficiencies with yeast harboring Myc-tagged Tfc1, Tfc4, Tfc6, Tfc7, or Tfc8 subunits of TFIIIC
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were also not substantially different (supplemental Fig. S1). The last result also argues against the possibility that the Myc tag exerts an effect on TFIIIC occupancy in vivo. The low absolute levels of TFIIIC occupancy at all transcriptionally active genes relative to TFIIIB and pol III occupancy (Table 3) strongly support the notion that TFIIIB suffices to recruit pol III for transcription in vivo as it does in vitro.

The exceptionally high occupancy of tRNA genes and SCR1 by TFIIIB and pol III (Table 3) suggests that these genes are set at maximal activity for transcription in yeast growing exponentially in rich medium. Maf1, the global repressor of pol III transcription, is associated with pol III-transcribed genes during repressive conditions; the low level of Maf1 occupancy that is maintained in exponentially growing cells is thought to fine-tune transcription by attenuation (19, 45). Elimination of Maf1 activity by deletion leads to a modest (0–36%) increase in pol III transcription in exponentially growing cells, analyzed in a small sample of genes (46, 47), and is correlated with at best modestly increased pol III and TFIIIB occupancy (19, 45). These small effects are consistent with the already high TFIIIB and pol III occupancies determined here. A much greater increase in tRNA gene transcription (∼3-fold) was observed in yeast harboring a temperature-sensitive Maf1 mutant (maf1–1) when grown at the restrictive temperature to late log phase in a low phosphate medium (48). If this stimulation of pol III transcription in the Maf1-defective cells reflects absolute levels of active transcription complexes in the corresponding wild type, this would indicate that Maf1-mediated repression under these growth conditions leaves only one-third of individual tRNA genes bearing active transcription complexes.

We have attempted to compare our TFIIIC, TFIIIB, and pol III occupancy determinations with the only qPCR analysis of ChIP enrichment (18) to examine more than one gene also represented in Table 3, along with nine tRNA genes that would provide a basis for scaling PCR-based relative enrichment to spiked complex-normalized occupancies. It should be noted that cells for the prior analysis were grown in synthetic medium, whereas the complex, rich medium YPD was used for this work. This difference in growth conditions may account for the extremely low relative occupancies of TFIIIC, TFIIIB, and pol III at SCR1 in the prior analysis compared with our observations. Roberts et al. (19) also reported relative ChIP enrichment of Bdp1 at SCR1 relative to ik(CU)U G1 to be 0.93 compared with 0.19 for the same genes in Moqtaderi and Struhl (18). Scaling on the basis of the average relative occupancy values for the nine tRNA genes analyzed in the prior work (18) to the average absolute occupancies of the five tRNA genes in Table 3 (the resulting raw occupancy scaling factors for Tfc4, Bdp1, and C34 are 0.22, 0.22, and 0.24, respectively) yields average TFIIIC/TFIIIB and pol III/TFIIIB occupancy ratios in the prior work (18) that are nearly identical with our observations (0.14 and 0.67, respectively, compared with 0.14 and 0.60 for genes listed in Table 3; excluding the ZOD1 and ETC loci in each case). Differences in growth conditions preclude definitive conversion of the prior data to quantitative occupancy, but a tentative assessment is provided in supplemental Table S1.

The notion that low TFIIIC occupancy, implied by lower ChIP enrichment, is a consequence of persistent transcription through the boxA- and boxB-binding sites of TFIIIC stems from the observation that, in the absence of TFIIIB, elongating pol III readily displaces TFIIIC from its binding sites in vitro (49). It has been shown that Maf1-mediated repression of pol III transcription results in decreased pol III occupancy on most of its conjugate genes (45), with a concomitant increase of TFIIIC occupancy at some genes (17, 19) but not others (45, 50). Gene-specific differences in response to transcriptional repression may reflect the transient nature of the increase in TFIIIC occupancy that was seen at the onset of repression by Roberts et al. (17, 19). Only Roberts et al. (17) examined the time variable (at three genes). Whether these transients are a common property of all pol III-transcribed genes remains to be determined. The transient nature of the TFIIIC occupancy change also raises the possibility that it may represent something more complex than a simple, direct response to a change of transcriptional traffic across its DNA-binding sites. It is also noteworthy that occupancy of ZOD1, which is essentially inert for transcription (unless activated by nucleosome depletion (18, 51)), by TFIIIC and TFIIIB is nearly the same (Table 3), yet the absolute level of TFIIIC occupancy is within the range of actively transcribed genes. Activation of this gene by nucleosome depletion was seen to have little or no effect on relative TFIIIC, TFIIIB, and pol III occupancy (51), yet ZOD1 is as susceptible to interference between ongoing transcription and TFIIIC occupancy as the tRNA genes (because its transcriptional terminator is sufficiently close to boxB to exclude simultaneous occupancy by pol III and the tα domain of TFIIIC).

We have attempted to force the issue of the relationship between TFIIIC occupancy and transcription by constructing an artificial SLIP4 tRNA gene (TA30pyr) that is unable to initiate transcription normally with a purine nucleotide, generating an at least 6-fold reduction relative to its reference construct, TA30 (Fig. 3B). The analysis had two unexpected outcomes. A minor surprise was finding a TA30pyr-dependent RNA 5′-end corresponding to bp +18, implying aberrant processing. (The more exciting alternative possibility that, when forced, pol III is capable of scanning a significant stretch of DNA in the downstream direction for an initiating purine is unlikely, because a start at bp +18 was not observed when this template was transcribed in vitro in the absence of tRNA processing; data not shown.) The principal surprise was that occupancy of the TA30pyr gene by TFIIIC and TFIIIB was dramatically reduced relative to TA30 (Fig. 3C). The site proximal pyrimidine substitutions in TA30pyr are unlikely to be directly responsible for this effect because they are far removed from the upstream binding site of TFIIIB and from the boxA-binding site of TFIIIC (and they also lie outside the boxA region of its DNase I footprint (16)), and TA30 and TA30pyr were equivalently competent in forming TFIIIB-TFIIIC-DNA and heparin-resistant TFIIIB-DNA complexes in vitro when assessed by EMSA (supplemental Fig. S2). The reduced formation of promoter complexes on TA30pyr (relative to TA30) is phenomenologically unlike Maf1-mediated repression, in that TFIIIC and TFIIIB occupancy diminishes more than pol III occupancy. In particular, the reduction of occupancy by the very stable TFIIIB-DNA complex to levels comparable with
those of pol III (Fig. 3C) suggests a dependence of TFIIIB-DNA complex maintenance on pol III.

pol III transcribes single-copy genes that are essential for cell viability (e.g. RPR1, SNR6). Although the stability of the TFIIIB-DNA complex is beneficial for robust activity of genes whose transcripts are required in abundance, it could be lethal if TFIIIB is damaged in such a way that the DNA binding capacity is maintained but its role in assembling pol III and/or facilitating open complex formation (52) is compromised. Misdirection of TFIIIB placement by TFIIIC (a phenomenon that is encountered in vitro (53, 54)) could also have lethal consequences. Thus, it would not be surprising to find that a mechanism exists for sensing TFIIIB-DNA complexes that are not associated with pol III and targeting them for disassembly. Such a pathway comes into play for yeast pol II transcription, with permanently arrested elongation complexes at sites of DNA damage being targeted for ubiquitylation and degradation (reviewed by Svejstrup (55)).

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