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# Genome-Wide Occupancy Profile of the RNA Polymerase III Machinery in *Saccharomyces cerevisiae* Reveals Loci with Incomplete Transcription Complexes†

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**We used chromatin immunoprecipitation, followed by microarray hybridization, to determine the genome-wide distribution of the RNA polymerase (Pol) III transcription apparatus in the yeast *Saccharomyces cerevisiae*. The Pol III transcriptome includes all tRNA genes, previously identified non-tRNA Pol III genes, and *SNR52*, which encodes a small nucleolar RNA. Unexpectedly, we identify eight *ETC* loci that are occupied by TFIIIC but not by other components of the Pol III machinery. Some *ETC* loci contain stretches of DNA that are highly conserved among closely related yeast species, suggesting that they may encode functional RNAs. *ETC6* is located upstream of the gene encoding the τ 91 subunit of TFIIIC, suggesting the possibility of Pol III-regulated expression of a critical Pol III factor. We also identify the *ZOD1* locus, which is bound by all components of the Pol III machinery and yet does not appear to express an RNA conserved among closely related yeast species. The B block motifs and several flanking nucleotides of the *ZOD1* and *ETC* loci are very similar to each other and are highly conserved across the yeast species. Furthermore, the unusual profile of Pol III factor association with *ZOD1* and the *ETC* loci is perfectly preserved in a different *Saccharomyces* species, indicating that these loci represent novel functional entities.**

Genes were originally defined more than a century ago by variants or mutations that alter the phenotype of an organism, and this method of identification remains useful today. Genes have also been defined by virtue of the RNAs or proteins they encode, and in many such cases, the phenotypes of mutations have not been assessed. With the advent of complete genomic sequences, genes are defined primarily by computational analysis. Although powerful, computational methods are necessarily biased by the specific properties used to define the genes. As a consequence, computational analyses often fail to identify real genes or, conversely, identify genes that are biologically meaningless. For example, as defined primarily by open reading frames (ORFs) greater than 99 amino acids, the yeast *Saccharomyces cerevisiae* was originally found to have 6,275 possible ORFs, 5,885 of which were predicted to be translated into proteins (9). Subsequent identification of transcribed regions of the yeast genome revealed 160 ORFs that had escaped annotation based on the original sequence-derived criteria (39). Recent computational studies based on sequence homologies among closely related yeast species suggest that approximately 500 of the originally defined genes are invalid, and they identify more than 40 smaller genes that were initially unrecognized (2, 19). Thus, in order to define the complete collection of genes in an organism, it is essential to use large-scale experimental approaches to complement the computational analysis.

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In eukaryotic organisms, RNA polymerase (Pol) III is responsible for the transcription of tRNAs and a few other nontranslated RNAs. Although greatly outnumbered by Pol II-transcribed, mRNA-encoding genes, Pol III genes are transcribed at very high frequencies and in fact constitute a much larger fraction of the total cellular RNA. In the yeast *Saccharomyces cerevisiae*, the non-tRNA genes transcribed by Pol III include the RNA component of RNase P (*RPR1*), the U6 small nuclear RNA (*SNR6*), the cytoplasmic RNA of the signal recognition particle (*SCR1*), and the 5S rRNA. In mammalian cells, the RNA component of RNase MRP is also transcribed by Pol III; its yeast counterpart *NME1* has not been identified as a Pol III gene. Several human genes, including the 7SK gene, the RNase MRP RNA gene, and the U6 RNA gene, have extragenic promoter elements and use a specialized Pol recruitment complex known as SNAPc (36), which is not found in yeast.

tRNAs and other Pol III genes share specific sequence and structural properties, including conserved A and B block sequences typically found within the coding region (7), thereby making it possible to search an entire genome for Pol III genes by computer-based methods. Such approaches revealed a total of 275 tRNA genes in the complete *S. cerevisiae* genome (10, 25, 32), as well as Pol III-transcribed *RNA170* (31). Northern analysis of long intergenic regions (31) and recent computational analysis based on conservation of secondary structure across species (26) have revealed a number of previously unpredicted noncoding RNAs. In addition, at least one as-yet-unidentified Pol III gene has been implicated in the processing of the major ribosomal (Pol I-dependent) transcript (13). Thus, it is unclear how many additional Pol III genes exist in the yeast genome.

The Pol III transcription apparatus consists of the three

multisubunit general transcription factors TFIIIB, TFIIIC, and RNA Pol III, with an additional factor, TFIIIA, playing a role in the transcription of the 5S rRNA (8). The assembly factor TFIIIC consists of six subunits, and it recognizes the conserved A and B blocks. These DNA sequence elements are usually found embedded within the RNA-coding sequence, though occasionally, as with *SNR6*, the B block may be located outside the transcribed region. TFIIIB, which is recruited by TFIIIC to a region upstream of the transcriptional start site, comprises the TATA-binding protein, TBP, the TFIIB-related factor Brf1, and the SANT domain protein Bdp1. RNA Pol III consists of 17 subunits, 10 of which are unique to Pol III, the others being common to two or all three of the yeast RNA Pols (8, 36).

We use chromatin immunoprecipitation, followed by microarray hybridization, to determine the genome-wide distribution of the Pol III transcription apparatus. The Pol III transcriptome in yeast includes essentially all tRNA genes, the previously identified non-tRNA genes, and *SNR52*, which encodes a small nucleolar RNA. These results are in agreement with recent reports that appeared after the work here was completed (11, 34). Unexpectedly, we identify *ZOD1*, a locus of unknown function associated with all components of the Pol III machinery, as well as eight *ETC* loci that are occupied by TFIIIC but not by TFIIIB or Pol III. The B-block sequences and association of the Pol III factors at these loci are conserved across *Saccharomyces* species, raising the possibilities of regulated Pol III recruitment and novel functions of the Pol III machinery.

## MATERIALS AND METHODS

**DNAs and yeast strains.** ZM356, which encodes $(HA)_3$ -Brf1, was created by inserting *BRF1* 5′ noncoding sequences upstream of a nuclear localization signal, triple hemagglutinin (HA) epitope tag, and an N-terminal fragment of the *BRF1* ORF in the *URA3* integrating vector pRS316. The wild-type gene encoding Brf1 was replaced by the gene encoding $(HA)_3$ -Brf1 in strain JD194 (5) by standard two-step gene replacement using plasmid ZMY356 to generate strain ZMY156. Experiments not requiring a tagged strain were performed in either ZMY156 or BY4741 (Research Genetics). *Saccharomyces mikatae* was obtained from Mark Johnston.

**Chromatin immunoprecipitation.** Cells were cultured in synthetic medium to an optical density at 600 nm of 0.6 to 0.8, and chromatin immunoprecipitation was performed essentially as described previously (21, 27) with antibodies against TBP, TFIIB, the HA epitope (F7; Santa Cruz Biotechnology), Bdp1 (kindly provided by Ian Willis), Tfc4, and Rpc34 (the latter two were kindly provided by Steve Hahn). Input and immunoprecipitated DNA samples were assayed by quantitative PCR in real time by using the Applied Biosystems 7700 or 7000 sequence detector. Occupancy values (in arbitrary units) were calculated by determining the apparent immunoprecipitation efficiency (i.e., the amount of PCR product in the immunoprecipitation sample divided by the amount of PCR product in the input sample) and dividing it by the apparent cross-linking efficiency of a control DNA segment (i.e., an internal fragment of the *POL1* structural gene and/or an inactive promoter). The resulting relative immunoprecipitation efficiency of the *POL1* control, 1.0, was then subtracted from all relative values to yield a background of zero.

**Microarray hybridization.** Microarrays were prepared with yeast intergenic sequence PCR products (Research Genetics) amplified by Matthew Copeland, and 6,528 PCR products were spotted in duplicate to polylysine-covered glass slides by Klaus Ullmann. A total of 25 to 50% of the DNA from the immunoprecipitated samples was amplified in the presence of 0.3 mM amino-allyl-dUTP (Sigma) and then labeled with Cy5 fluorescent dye (Amersham Biosciences) essentially as described previously (16). The input DNA samples were similarly amplified and labeled with Cy3 dye. A mixture of labeled immunoprecipitation sample and control DNA was applied to each microarray, followed by incubation at 45°C overnight, before being washed by standard methods (16). Slides were scanned on an Axon scanner, and the data were analyzed by using Axon GenePix 4.0 software. Each microarray experiment was performed a minimum of three times.

Further data analysis was performed in Microsoft Excel. All spots flagged by the GenePix software as either poor-quality or undetectable were removed from the analysis. The data were normalized within each experiment (and across all replicates of the experiment) such that the background-subtracted median fluorescence for Cy5 over the entire array was equal to that of Cy3. The ratio of Cy5 to Cy3 was calculated for each spot, and the median ratio for each spot was then computed across all replicates of the experiment (see the supplemental material). Because each array was spotted in duplicate and at least three arrays were used per sample, a minimum of six values contributed to the median for each spot. Spots were then ranked in descending order by median Cy5/Cy3 ratio. Research Genetics intergenic sequence names were then cross-referenced with gene, and feature names were obtained from the *Saccharomyces* Genome Database (SGD) (14).

**Sequence analysis.** Sequence comparison across *Saccharomyces* species was performed by using the fungal alignments tool of the SGD. The AlignAce program (35) was used to search for common sequence motifs across multiple loci. The Pattern Matching tool of the SGD was used to search for sequences matching the derived *ETC* consensus.

**RNA quantitation.** Total RNA was isolated as described previously (15) and then further purified by using Qiagen RNeasy columns with DNase I treatment. First-strand cDNA was synthesized by using oligo(dT) or random hexamer primers, and quantitative PCR in real time was performed on the resulting first-strand cDNA with primers specific to the gene of interest (33).

## RESULTS

**Genome-wide association of Pol III transcription factors.** To obtain a genome-wide profile of occupancy by the Pol III machinery, we investigated DNA association in vivo by proteins representing each of the three multisubunit Pol III-specific transcription factors TFIIIB, TFIIIC, and Pol III. Chromatin immunoprecipitation was performed on cells grown in synthetic complete medium with antibodies against the $(HA)_3$-tagged TFIIIB subunit Brf1, the TFIIIB subunit Bdp1, the TFIIIC subunit Tfc4, and the Pol III subunit Rpc34. Input and immunoprecipitated samples were amplified and then applied to a microarray spotted in duplicate with 6528 PCR products corresponding to almost all yeast intergenic sequences. A fraction of the nonamplified immunoprecipitated material from each experiment was reserved for quantitative analysis of specific target genes.

With a few notable exceptions (see below), the occupancy profiles of the four factors are extremely similar (Fig. 1). To assess the relative occupancies by the different factors, we analyzed the 1,200 spots for which at least one of the four factors is present at least twofold over the median ratio. When the median ratio of Bdp1 occupancy to Brf1 occupancy at these 1,200 spots is set to 1.0, the standard deviation of values around this median is 0.63, indicating good cooccupancy, as expected, by these two subunits of TFIIIB. Bdp1 occupancy is also well correlated with Rpc34 occupancy, with a standard deviation of 0.75. The Tfc4 distribution is considerably broader; when the median ratio of Tfc4 occupancy to Rpc34 is set to 1.0, the standard deviation is 1.98. Similarly, the standard deviation of the Tfc4 to Bdp1 ratio is 2.26. Thus, although Tfc4 is required by all Pol III genes, the amount of Tfc4 relative to other factors is somewhat variable across different Pol III genes (several extreme examples will be discussed in a later section). These observations suggest that, in general, the Pol III factors associate with genomic sequences in the context of the intact Pol III machinery.

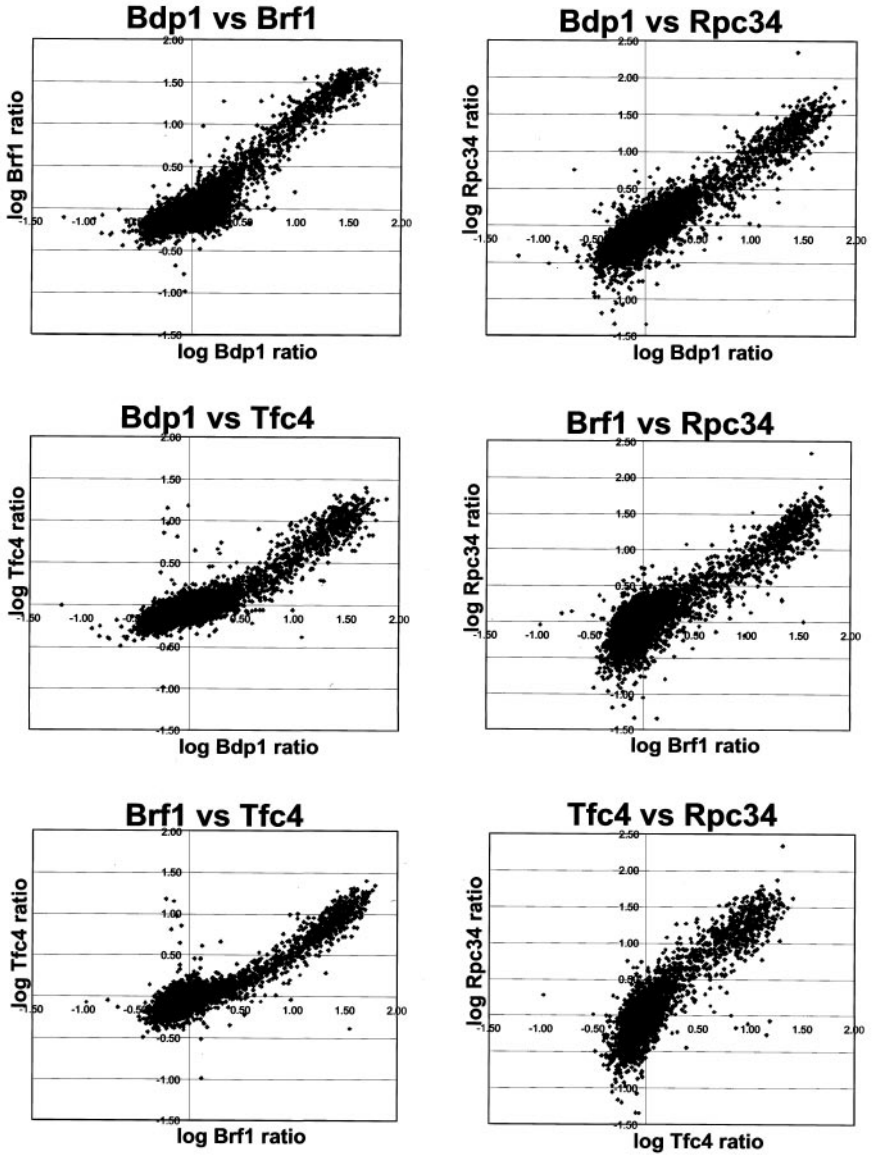


FIG. 1. Pairwise comparisons of occupancy by the indicated Pol III factors. Each point represents the occupancy values of the two indicated factors at an individual microarray spot. The occupancy value is the log of the median ratio of immunoprecipitated DNA signal to input DNA signal.

**The Pol III machinery associates with all tRNA genes, but with a fivefold range in occupancy.** As a first step to define targets of the Pol III machinery, we identified 633 spots on the array that yield immunoprecipitation/input ratios that are at least twice the median for all four factors. As expected, the targets of the Pol III machinery are overwhelmingly tRNA promoters or sequences adjacent to the 3′ termini of tRNAs. Because of the small size (71 to 133 bp) of tRNA genes and the resolution limitations of the array experiment, we obtained an extremely high signal for any sequence abutting a tRNA gene, regardless of whether it is upstream or downstream of the tRNA gene. A given tRNA therefore typically contributes to a positive signal in at least two spots. In a few instances, several closely spaced tRNAs are represented by a single spot on the microarray; their individual contributions to the occupancy value of the spot cannot be distinguished by the array alone. A total of 232 tRNAs are represented by distinct spots on our array and yielded high-quality data for all replicates of the four factors we tested. Of these, 217 (94%) yielded a Pol III signal in the top 10% of microarray spots in our overall ranking. Among these is the *tX(XXX)D* tRNA gene, for which no anticodon has been annotated at SGD. Three tRNAs are unexpectedly low (in the bottom third) on our ranked list of Brf1-occupied sequences, but direct analysis reveals these to be significantly associated with Pol III transcription factors and, hence, false negatives on the array. Taken together, our results indicate that the Pol III machinery associates with all tRNA genes.

Although the array experiments are suggestive of a spectrum of occupancy levels, the spotted PCR products vary in size and

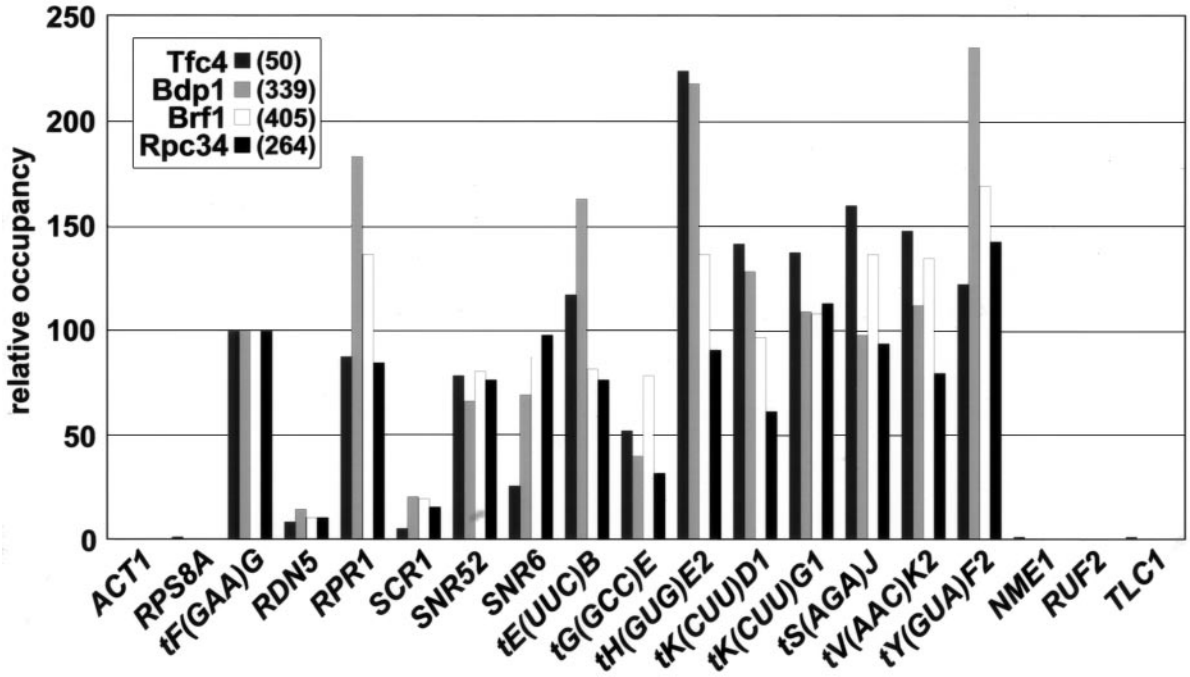


FIG. 2. Relative occupancy of various genomic regions by Pol III factors. Cross-linked chromatin was immunoprecipitated with antibodies against the indicated proteins, followed by quantitative PCR in real time with primers to the indicated genomic regions. All occupancies were measured relative to a control DNA representing the center of the *POL1* coding region, and the results are shown scaled such that the occupancy value of each factor at *tF(GAA)G* equals 100. The actual raw occupancy value (relative to the *POL1* control) for each factor at *tF(GAA)G* is shown in the inset. The apparently low value for Tfc4 at *SNR6* is likely to reflect the downstream location of the B block with respect to the primer pair used in the analysis.

are often considerably longer than a tRNA, with the result that any positive occupancy signal is diluted to various extents by the unbound neighboring DNA in the same PCR product. We therefore used quantitative PCR with primers targeted to individual small regions of DNA to measure the occupancy of Pol III factors at a variety of Pol III genes (Fig. 2). All four factors yield exceptionally robust occupancy values, with Bdp1, Brf1, and Rpc34 typically enriched >200-fold at Pol III promoters relative to the control DNA; even the weakest immunoprecipitation results, those of Tfc4, averaged well over 20-fold enrichment at the Pol III promoters. The difference in enrichment values between the Tfc4 immunoprecipitation and those of the other factors may be attributable to a combination of different cross-linking efficiencies and differences in the antibodies.

For all tRNA promoters tested, the relative associations of the different components of the Pol III apparatus were fairly constant, strongly suggesting that the intact Pol III machinery is present and required for stable association. This observation is similar to that observed for basal factors involved in Pol II transcription (e.g., TBP, TFIIA, TFIIB, and Pol II) but different from that observed with TBP-associated factors, which can be relatively high or low depending on the promoter (20–23). The overall level of the Pol III factors at different tRNAs varies over a considerable range. To take an extreme example, at *tY(GUA)F2*, Bdp1 occupancy is approximately seven times greater than it is at *tG(GCC)E*, and Rpc34 occupancy is approximately five times higher (Fig. 2). Occupancy of typical tRNA genes (defined by six of the nine genes individually tested) by Pol III factors averages ca. 60% of the maximal observed level. Although four tRNAs [*tD(GUC)K*, *tI(UAU)D*, *tI(UAU)L*, and *tP(UGG)F*] can be transcribed in vitro in the absence of TFIIIC (6), association of Tfc4 at these promoters in vivo occurs at a level commensurate with that found at other tRNAs. This finding is in accord with the observation that *SNR6* requires TFIIIC for transcription in the context of chromatin (1), even though it can be transcribed in vitro by a TFIIIC-independent mechanism (17).

**Occupancy of the 5S and *SCR1* genes by Pol III factors is low compared to typical tRNA genes.** In addition to the tRNAs, genes encoding all other previously known Pol III-transcribed RNAs (*RDN5*, *RPR1*, *SCR1*, and *SNR6*) show a high level of occupancy by Pol III factors. We observe no significant Brf1 occupancy at *NME1* or *TLC1*, two genes encoding nontranslated RNAs. Pol III factors are also not associated with the *RUF* genes, which encode a recently described group of non-protein-coding RNAs (26). In contrast, *NME1*, *TLC1*, and the *RUF* genes show high occupancy by the Pol II-specific transcription factor TFIIB (data not shown), confirming that these genes are transcribed by Pol II in *S. cerevisiae*.

The occupancy levels of these Pol III factors at *RPR1* and *SNR6* are similar to that of typical tRNA promoters, whereas the 5S rRNA gene (*RDN5*) associates with almost 10-fold-lower levels of Brf1, Bdp1, and Rpc34 than a typical tRNA. This may be at least partially explained by the fact that there are more than 100 tandem copies of the ribosomal DNA in yeast, of which only half are actively transcribed (4). If 50% of the 5S copies are fully active, with the remaining 50% com-

pletely inactive, then Pol III occupancy of active 5S copies is actually fivefold lower than the level at a typical tRNA (Fig. 2). Our results cannot exclude a scenario in which a low level of Pol III is present at every 5S copy, but this is unlikely because factor occupancy is highly correlated with transcription. The lower Pol III occupancy at the 5S gene might also reflect less efficient recruitment of other Pol III factors by TFIIIA.

The *SCR1* gene is associated with approximately fivefold lower amounts of Brf1, Bdp1, and Rpc34 than a typical tRNA, and Tfc4 occupancy at this locus is only 5% that of an average tRNA (Fig. 2). *SCR1* is unusual in that it is far longer (522 nucleotides [nt]) than all other known yeast Pol III transcripts. However, *SCR1* has both A and B blocks in typical intragenic positions, and it assembles all components of the Pol III apparatus, although apparently less efficiently than most tRNA genes.

***SNR52* is a Pol III gene.** In attempting to identify previously unidentified targets of the Pol III machinery, we observed that only 13 of the 500 genes with the highest Brf1 occupancy are not adjacent to a known Pol III gene or to a retroelement. Retroelements are present in multiple copies, many of which are adjacent to tRNAs, and they may cross-hybridize with other copies in non-tRNA-containing spots. All 13 potential sites of Pol III occupancy were tested by real-time quantitative PCR. With two exceptions (*SNR52* and *ZOD1*, see below), we found no occupancy of these loci by Pol III factors, indicating that most of these spots are false positives on the microarray (most likely due to incorrect PCR products). Spots ranked lower on the Brf1 array, between 500 and 800, often represent loci that, although not immediately adjacent to Pol III genes, are very close to tRNAs, with only very small intervening genes. We tested 18 other genomic locations within this range for which there is no Pol III gene nearby; all were negative by quantitative PCR. These results suggest that there are few, if any, other Pol III-associated loci on our arrays.

One previously unsuspected site of Pol III occupancy is *SNR52*, which encodes a small nucleolar RNA involved in ribose methylation of rRNA (24). Due to the proximity of *SNR52* to tRNA *tH(GUG)E2*, we mapped the location of the Pol III machinery along this chromosomal region (Fig. 3). A distinct peak of Pol III factor occupancy is observed over *SNR52*, at a level similar to that of *SNR6*. Occupancy of the Pol III machinery is specific to *SNR52*, as can be seen by the sharp drop in occupancy before a second specific Pol III peak appears over *tH(GUG)E2*. *SNR52* RNA levels are comparable to those of other Pol III transcripts (data not shown), and there is minimal occupancy by the Pol II transcription factor TFIIB over the *SNR52* promoter (data not shown). The *SNR52* locus contains the sequence GTTCGAAAC 35 bp upstream of the start of the mature RNA coding sequence; this sequence corresponds fairly well to the B block consensus GTTCRANYC (7), and its position is similar to that of the B block 30 bp upstream of the mature *RPR1* RNA (23). Thus, *SNR52* is a previously unsuspected Pol III-transcribed gene.

***ZOD1*, a functional Pol III promoter.** Our array results indicate significant occupancy by all four Pol III factors in the intergenic region upstream of *UFO1*, a gene encoding an F-box protein required for the ubiquitin-mediated degradation of the HO endonuclease (18). DNA sequencing of a PCR-amplified region from position −447 to position +165 with respect to the

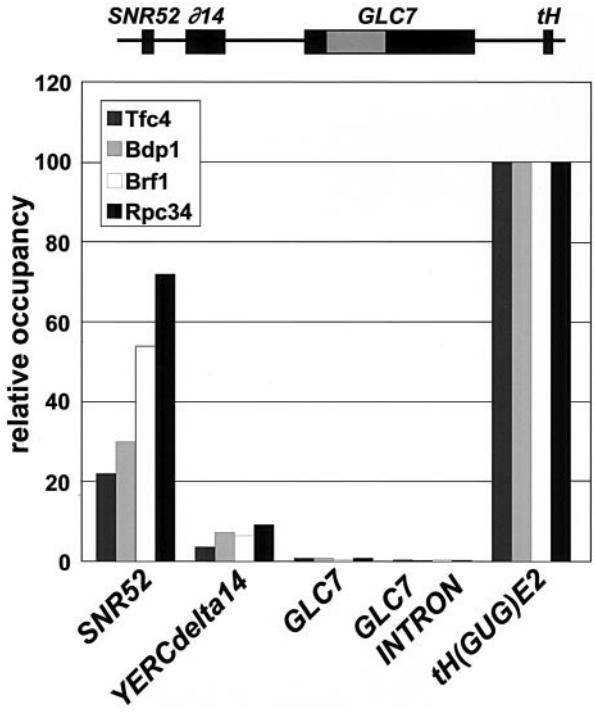


FIG. 3. *SNR52* is a Pol III-transcribed gene. Relative occupancy by the indicated Pol III transcription factors across the *SNR52-YERCΔ14-GLC7-tH(GUG)E2* region, with values at *tH(GUG)E* set to 100.

*UFO1* translational start site shows this sequence in our strain to be identical to the published sequence, thus eliminating the trivial possibility that a tRNA might be present in this region in our particular strain (data not shown). In addition, all Pol III factors associate with this region in two different *S. cerevisiae* strains (data not shown). Mapping experiments with a set of tightly spaced primer pairs reveals coincident peaks of Brf1, Bdp1, Tfc4, and Pol III occupancy between positions −344 and −252 with respect to the *UFO1* translational start site (Fig. 4). Interestingly, occupancy by Tfc4 is relatively high at this locus, and occupancy by Pol III is disproportionately low, yielding an unusual Tfc4/Rpc34 ratio that is 15.5-fold higher than that of *tF(GAA)G*. For this and other reasons to be described below, we term this locus *ZOD1* (for zone of disparity).

As expected from the occupancy of Pol III factors, *ZOD1* contains putative A and B blocks whose spacing is similar to that found in Pol III-transcribed genes. The sequence GGTT CGAACTC at position −205 relative to the translational start of *UFO1* is a good match to the B-block consensus, and the sequence TTGGCGCTTTGG at position −237 is a fairly good match to the consensus for the A block. Taken together, our results indicate that *ZOD1* encodes a functional Pol III promoter, i.e., one that assembles a complete transcription apparatus.

***ETC* loci that are occupied by TFIIIC but not by other Pol III factors.** As mentioned above, pairwise comparisons of the genomic profiles and quantitative analysis of individual genes indicate that the association levels of the Pol III factors are strongly correlated. Unexpectedly, seven loci representing the upstream regions of *TFC6-ESC2*, *ADE8-SIZ1*, *ARG8*, *BCK1*, *RAD14-ERG2*, *RAD2*, and *WTM2-YOR228C* (gene names sep-

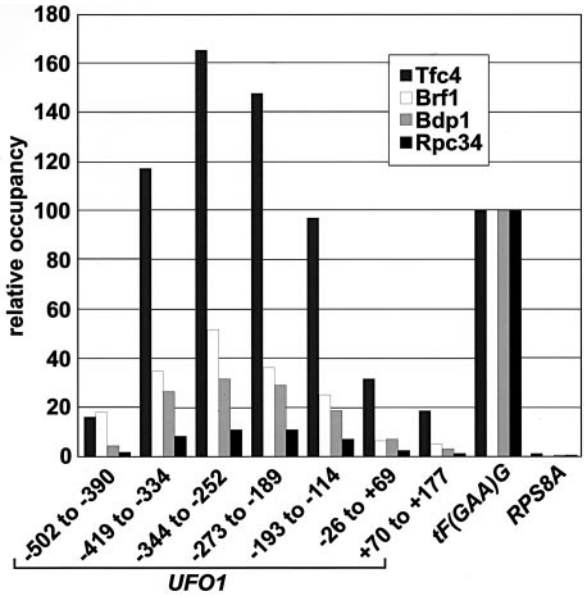


FIG. 4. *ZOD1* is bound by the intact Pol III machinery. Cross-linked chromatin was immunoprecipitated with antibodies against the indicated Pol III factors, followed by quantitative PCR in real time. The tightly spaced primer pairs correspond to the indicated genomic regions upstream of *UFO1* (defined with respect to the translational start at position +1). All values are shown relative to the occupancy at *tF(GAA)G*, which was set to 100.

| LOCUS | B BLOCK ALIGNMENT | Most conserved stretch | |
|---|---|---|---|
| *ZOD1 (UFO1)* | CCG**GTTCGAACTC**GGGGT**GAGGC** | 77/122 | (63%) |
| *ETC1 (ADE8-SIZ1)* | **CTCATTCGA**ATCCTTG**CTGACGC** | 67/88 | (76%) |
| *ETC2 (ARG8)* | **CCATATCGA**ATCCC**GATAGGAGC** | 56/67 | (84%) |
| *ETC3 (BCK1)* | G**CCATTCAATTC**CAGAC**CGACGC** | 43/74 | (58%) |
| *ETC4 (RAD2)* | C**CCATTCGAA**CCTCCGT**GGAGGC** | 54/86 | (63%) |
| *ETC5 (RNA170)* | GT**GGTTCGATTCTGC**CCT**GGAGC** | 118/170 | (69%) |
| *ETC6 (TFC6-ESC2)* | **GCGGTTCGAAA**ACCCTA**CGTTGC** | 78/113 | (69%) |
| *ETC7 (WTM2-YOR228C)* | G**CAGTTCGAACCCC**GAAC**GGGGC** | 100/113 | (89%) |
| *ETC8 (RPB5-CNS1)* | **TCTATTCGACTCC**TAAC**GGAGGC** | 54/86 | (63%) |
| B block consensus | GGTTCGANYCC | | |
| *ETC* consensus | $C_NRTTCGAAYCC_{NNN}R_NYGR_NGC$ | | |
| yeast tRNA consensus | $_{NN}GGTTCGA_NYCY_{NNNNNNNN}R_NC$ | | |

FIG. 6. Evolutionary conservation of B blocks and nearby regions of *ZOD1* and *ETC* loci across *Saccharomyces* sensu stricto yeast. The putative B blocks and immediately flanking sequences of *ZOD1* and the eight *ETC* loci are aligned to the B block consensus. The neighboring Pol II-transcribed gene(s) whose 5′-noncoding regions are adjacent to each locus are indicated (hyphens indicate divergently transcribed genes). Shown below is the consensus derived from *ZOD1* and *ETC1* to *ETC7*, as well as the B block consensus derived from the analysis of 274 *S. cerevisiae* tRNAs. For each derived consensus, the height of each letter is directly proportional to its degree of identity across the contributing sequences. Nucleotides in bold are identical across the four yeasts *S. cerevisiae*, *S. bayanus*, *S. mikatae*, and *S. paradoxus*. For each locus, the most highly conserved region near the B block across these four yeast species is shown as a percentage of identical nucleotides spanning the length specified.

arated by a hyphen indicate that the locus is between these two divergently transcribed genes) are occupied by Tfc4 but not occupied to a significant extent by the other Pol III factors tested. Quantitative analysis (Fig. 5) indicates that the levels of Tfc4 association at these seven atypical loci (14- to 35-fold enrichment relative to the background) are roughly comparable to those at other Pol III genes. In contrast, occupancy by Bdp1, Brf1, and Rpc34 is not observed, even though typical Pol III genes exceed 200-fold enrichment above background for these factors. One of these loci, the intergenic region between *RAD14* and *ERG2*, contains the gene encoding *RNA170*, a Pol III RNA of unknown function (31); the others have not been identified in any screen for noncoding RNAs or Pol III genes. We have designated these loci *ETC* (for extra TFIIIC) (Fig. 6). An eighth *ETC* locus, in the *RPB5-CNS1* intergenic region, was identified by sequence analysis (see below).

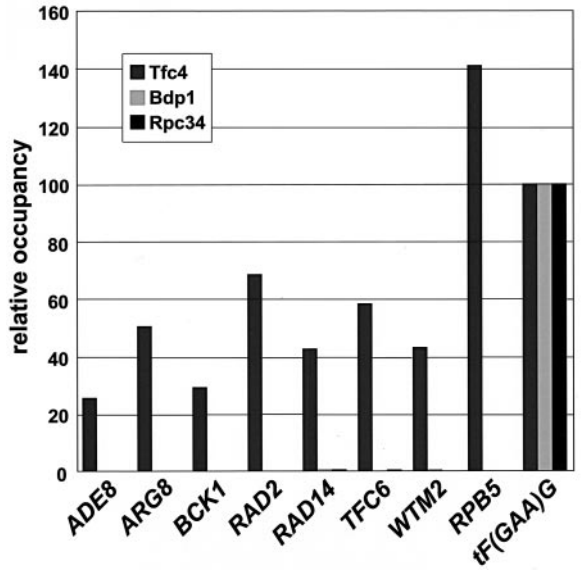


FIG. 5. *ETC* loci are bound by TFIIIC but not by TFIIIB or Pol III. Cross-linked chromatin was immunoprecipitated with antibodies against the indicated proteins, followed by quantitative PCR in real time with primers to the indicated genomic regions. All occupancy values are scaled to set the occupancy at *tF(GAA)G* equal to 100.

***ZOD1* and the *ETC* loci contain B blocks and additional conserved residues.** Although *ZOD1* differs from the *ETC* loci in that it recruits the complete Pol III apparatus, the disproportionately high Tfc4 occupancy at *ZOD1* is reminiscent of the *ETC* loci. We therefore examined all eight loci for common sequence elements. The AlignAce motif-finding program (35) reveals a common sequence resembling an extended B block in all eight of these intergenic regions, with a maximum a posteriori score 18 and a specificity score of 2.8 $e^{-12}$. Seven of these sequences are high-quality matches to the B block consensus, and the more degenerate sequence at *ETC3* is also aligned (Fig. 6). Interestingly, there is 100% conservation of three additional nucleotides located 6 to 10 bases downstream of the B block consensus (Fig. 6). Alignment of the B blocks of 274 *S. cerevisiae* tRNA genes indicates that one of these bases, the final C, is significantly conserved in tRNA sequences (204 instances out of 274 tRNAs). Perfect conservation of all three nucleotides is found in only 21 of 274 tRNAs but at all *ETC* loci. The presence of a B-block makes it likely that TFIIIC association at these loci occurs in a manner generally similar to its association with typical Pol III genes. At the same time, the

perfect conservation of three extra nucleotides suggests that there is something special about these otherwise apparently normal TFIIIC interaction sites.

A search of yeast intergenic sequences for loci matching the derived *ETC* consensus (CNRTTCGAAYCCNNRNYGRN GC), allowing for two mismatches, revealed two additional matches in loci not adjacent to tRNAs. Quantitative analysis indicates that the region upstream of *SIP1* does not associate with any Pol III factors (data not shown), whereas the region upstream of *RPB5-CNS1* displays the Pol III occupancy profile of an *ETC* locus (Fig. 5). We termed this locus *ETC8*; its alignment with *ZOD1* and *ETC1* to *ETC7* is shown in Fig. 6.

**Phylogenetic conservation of *ETC* and *ZOD1* sequences.** Functionally meaningful sequences tend be highly conserved across *Saccharomyces* species, with protein-coding sequences having a much higher level of cross-species identity than intergenic sequences (2, 19). Interestingly, the B block of each *ETC* locus is highly conserved among the four *Saccharomyces* sensu stricto yeasts: *S. cerevisiae*, *S. mikatae*, *S. bayanus*, and *S. paradoxus*. Of the 11 positions within the B block consensus, 8 to 10 positions are identical (the boldface residues in Fig. 6) in the four yeasts, and many of the nonidentities are at positions that are less conserved among B blocks in *S. cerevisiae*. In addition, the three extra nucleotides downstream of B block core that are characteristic of the *ZOD1* and *ETC* loci are extremely well conserved across the four yeast species. This striking conservation suggests that Tfc4 is likely to associate with these regions in the other yeast species and that Tfc4 association is biologically meaningful.

In general, the cross-species identity of Pol III-transcribed RNAs within the genus *Saccharomyces* is fairly high. For example, the sequence identity across the four *Saccharomyces* sensu stricto yeasts is 79% for *RPR1*, 86% for *SCR1*, and 100% for *tC(GCA)P2*. *RNA170*, which corresponds to *ETC5*, has 70% identity if the RNA is defined by the further upstream of its two 3′ termini; however, the next 70 nt until the downstream 3′ end are only 21% conserved. At the remaining seven *ETC* loci, sequence conservation across these yeasts is variable. Conservation of *ETC4* (63% identity over 86 nt near the putative B block) and *ETC3* (58% identity over a 74-nt region) is below that of all known RNA genes, making it unclear whether these loci encode a defined RNA species. In contrast, *ETC7* has a 113-nt region with 89% identity across the four species, and *ETC2* has a 67-nt region with 84% identity. This very high degree of sequence conservation suggests that *ETC2* and *ETC7* might encode heretofore undescribed RNAs transcribed by Pol III.

For *ZOD1*, the putative B block and 8 of 12 bases of the putative A block are identical across the *Saccharomyces* sensu stricto species. The presence of highly conserved and appropriately spaced A and B blocks, together with occupancy by Pol III factors, is suggestive of a biologically relevant Pol III function, although we cannot exclude the possibility that these conserved sequences are involved in a *UFO1*-related function. In contrast to the A and B blocks, sequences corresponding to the peak of Pol III, TFIIIC, and TFIIIB occupancy (positions −200 to −400 with respect to the *UFO1* translation start) are only 26% identical, indicating that this region does not encode a conserved RNA. A 122-nt region closer to *UFO1* (positions −165 to −44) is 63% identical across these yeasts, but this region corresponds poorly to the location of Pol III and thus may represent *UFO1* regulatory sequences. Hence, *ZOD1* contains highly conserved A and B blocks yet may not encode a defined RNA species.

**Functional properties of *ZOD1* and the *ETC* loci are conserved in another *Saccharomyces* species.** To address whether *ZOD1* and the *ETC* loci represent fortuitous binding sites of Pol III factors or a conserved biological function, we examined occupancy of Pol III factors at these loci in *S. mikatae*. Exploiting the significant protein sequence identity across *Saccharomyces* sensu stricto yeasts, we used antibodies against *S. cerevisiae* Bdp1, Tfc4, and Rpc34 to immunoprecipitate chromatin from *S. mikatae*. The occupancy profiles of the *S. mikatae ETC* and *ZOD1* loci (Fig. 7) are extremely similar to those observed in *S. cerevisiae*, including the skewed TFIIIC/Pol III ratio at *ZOD1*. Just as in *S. cerevisiae*, high occupancy by Tfc4 at the *ETC* loci in *S. mikatae* is unaccompanied by significant Bdp1 or Pol III occupancy. Interestingly, *ETC5*, the only *ETC* locus that coincides with a known *S. cerevisiae* RNA (*RNA170*), may show a very slight occupancy by Bdp1 and Pol III in *S. mikatae*. The striking functional similarities of *ZOD1* and the *ETC* loci in *S. cerevisiae* and *S. mikatae* strongly suggest that these loci possess conserved biological functions.

**Attempted identification of a *ZOD1* RNA.** Given the apparent disparity between the highly conserved A and B boxes and the lack of sequence conservation in the region of high occupancy by Pol III factors, we used two methods to address whether *ZOD1* encodes a defined RNA species. First, randomly primed, reverse transcriptase PCR with the closely spaced primer set described above revealed the expected *UFO1* transcript but no RNA corresponding to the region occupied by Pol III factors. The spacing of our PCR primers is such that we would have observed only longer transcripts that are at least 100 nt. Second, we performed S1 nuclease protection on total RNA with overlapping oligonucleotide probes (55 to 71 nt in length) complementary to both strands across the region from positions −491 to −60 relative to the *UFO1* translational start site. This method yielded no evidence of discrete RNA transcripts, although this approach requires that a transcript contain approximately 35 (or more) bases of homology from the 5′ end of a given oligonucleotide probe. Thus, we have been unable to detect a significant *ZOD1* transcript, although there are some limited locations for a small transcript that might have been missed due to the placement of the probes used in the analysis.

## DISCUSSION

We have defined the Pol III transcriptome in *S. cerevisiae* by determining the genome-wide distribution of the Pol III transcription apparatus, which comprises TFIIIC, TFIIIB, and Pol III itself. The Pol III transcriptome includes all tRNA genes, previously identified non-tRNA Pol III genes, and *SNR52*, which encodes a small nucleolar RNA. During the preparation of this paper, similar results were reported elsewhere (11, 34). In addition, our results define nine Pol III-related loci with unusual properties. As discussed below, these include eight *ETC* loci that associate with TFIIIC but not with TFIIIB or Pol III, as well as *ZOD1*, which is bound by all components of the Pol III machinery but may not encode a discrete RNA species.

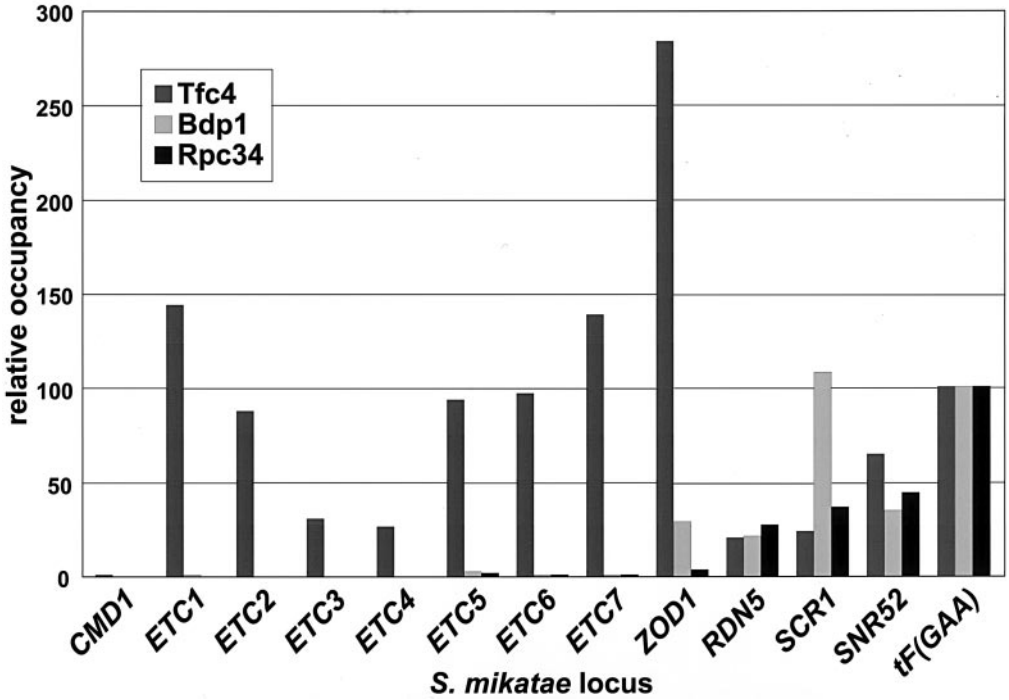


FIG. 7. The Pol III factor occupancy profiles of *S. mikatae ZOD1* and *ETC* loci parallel those of *S. cerevisiae*. Cross-linked chromatin from *S. mikatae* was immunoprecipitated with antibodies to the indicated Pol III factors and analyzed by quantitative PCR in real time with primers specific to the indicated regions of *S. mikatae* DNA. All occupancy values shown are relative, with the value for each factor at *tF(GAA)* set to 100.

***ZOD1*, a Pol III promoter that may not generate a discrete and functional RNA.** *ZOD1* is defined as a functional Pol III promoter because it associates with all components of the Pol III apparatus at levels observed at other Pol III genes. The Pol III promoter at *ZOD1* is biologically meaningful because the sequence and spacing of the A and B blocks are highly conserved in the four *Saccharomyces* sensu stricto species, and the Pol III occupancy of this locus is preserved in a *S. mikatae*. However, we have been unable to detect any RNA associated with *ZOD1*, and the region occupied by the Pol III machinery is not conserved among the related yeast species. We consider three models for *ZOD1*.

First, *ZOD1* might be a promoter that is bound by a transcriptionally incompetent form of the complete Pol III machinery. Although a promoter-bound Pol III machinery is transcriptionally inactive in vitro in the absence of nucleotide precursors, there is no evidence for a factor that is necessary for the transition between assembly of a complete preinitiation complex and transcriptional initiation. In vivo, the Pol III machinery actively transcribes numerous genes; hence, it is highly unlikely that the Pol III machinery per se is specifically inactive at *ZOD1*. However, it is possible that *ZOD1* contains a positioned nucleosome that permits binding by the Pol III machinery but blocks significant elongation, since proper nucleosome positioning can be critical for the transcription in vitro (38). Alternatively, a specific DNA-binding protein might block elongation.

Second, *ZOD1* might encode a discrete and functional RNA species, similar to those of standard Pol III-transcribed genes. Although we were unable to detect such an RNA, our analysis would probably not detect RNAs less than 35 nt in length or somewhat longer RNAs that map at certain locations. It is unlikely that a *ZOD1* RNA species is undetectable simply because it is poorly expressed. The assays used are capable of detecting less than one molecule/cell (15), occupancy by transcription factors is highly correlated with transcription levels (21, 23), typical Pol III transcript levels are very high, and occupancy by the Pol III apparatus at *ZOD1* is comparable to that of other Pol III-transcribed genes. Importantly, with the exception of the B block, the *ZOD1* region occupied by the Pol III machinery is not conserved among the four *Saccharomyces* strains. There is some sequence conservation in the region more proximal to the adjacent Pol II-transcribed *UFO1* gene, but this region does not coincide with the location of the Pol III factors. Thus, any discrete Pol III-transcribed *ZOD1* RNA that escaped detection would have to be either unusually distant from the B block or nonconserved, even though the A and B blocks are highly conserved.

Third, the Pol III machinery at *ZOD1* might generate short, nonsensical transcripts that are unstable. This scenario might be expected when a functional Pol III promoter is located in a nonconserved region. The *ZOD1* region is riddled with poly(dT) stretches, which can act as termination signals for Pol III; any resulting short nonfunctional RNAs are likely to be degraded and hence difficult to detect. Although this model might imply that *S. cerevisiae ZOD1* is a meaningless pseudogene or other genomic relic, the high conservation of the *ZOD1* A and B blocks, as well as the conserved binding of Pol III factors to this locus in *S. mikatae*, suggest that any localized Pol III transcription is likely to have a physiological function.

***ETC* loci represent partial Pol III transcription complexes that are likely to be physiologically significant.** TFIIIC is the

major promoter-recognition factor of the Pol III machinery, and it binds the A and B blocks in vitro in the absence of other Pol III factors (8). TFIIIB and Pol III are recruited by TFIIIC to form stable Pol III transcription complexes. Although TFIIIC association strongly correlates with the association of the entire Pol III machinery, the existence of the eight *ETC* loci indicates that bound TFIIIC is not sufficient to recruit TFIIIB and Pol III to promoters in vivo. In this regard, when cells approach stationary phase, TFIIIC remains bound to most Pol III genes, whereas recruitment of Pol III and TFIIIB is clearly reduced (11, 34). This growth-regulated recruitment of Pol III and TFIIIB is a general feature of Pol III genes; in contrast, the *ETC* loci exhibit a factor recruitment profile distinct from that of typical Pol III promoters. This strongly suggests that Pol III promoters contain DNA sequence determinants distinct from the A and B blocks that are important for association of TFIIIB and/or Pol III. It is possible that the three conserved bases near the B block are important for the functional differences of these loci from typical Pol III genes.

We considered four models, not mutually exclusive, for how TFIIIC, but not TFIIIB and Pol III, associates with these *ETC* loci. First, *ETC* loci might lack functional TATA-like sequences just upstream of the RNA initiation site, given that such sequences can influence Pol III transcription in vivo (12) and in vitro (40), presumably by facilitating association of TFIIIB (3, 37). However, many Pol III promoters lack canonical TATA elements, and the transcriptional effects of the TATA-like sequences are quantitatively modest. Second, the *ETC* loci might have DNA sequences that result in positioned nucleosomes that do not interfere with TFIIIC binding but essentially block association of TFIIIB and Pol III. Third, DNA-binding proteins bound at critical positions at the *ETC* loci might block the association or recruitment of TFIIIB and Pol III while not affecting association of TFIIIC. Fourth, TFIIIC bound at the *ETC* loci may be in a conformation that precludes its association with TFIIIB. In this regard, the Brf1-interacting region of the TFIIIC subunit Tfc4 is subject to autoinhibition, and a dominant Tfc4 mutation can cause a conformational change in Tfc4 that promotes a more favorable TFIIIC-TFIIIB interaction (28–30). For the last two models, the three conserved residues flanking the B block might play some role.

From a biological perspective, the *ETC* loci may be false Pol III promoters that could be derived from vestigial Pol III genes or could be fortuitous occurrences of TFIIIC recognition sequences. A more likely possibility, especially in light of the sequence conservation among related yeast species and the functional conservation of Tfc4 binding in *S. mikatae*, is that some (and perhaps all) *ETC* loci are bona fide Pol III promoters at which the complete Pol III apparatus assembles only under specific conditions. *ETC5* appears to be linked to the Pol III transcript *RNA170* (31), and *ETC2* and *ETC7* are linked to highly conserved regions typical of functional RNA species. Perhaps a modification of TFIIIC or TFIIIB in response to a specific environmental or genetic condition might permit the recruitment of the intact Pol III machinery to the *ETC* loci. Alternatively, a given condition might result in the dissociation of an inhibitory factor or disruption of a positioned nucleosome that blocks TFIIIB and Pol III entry at these loci; this could possibly have implications for the regulation of the neighboring genes. It is intriguing that *ETC6* is located upstream of *TFC6*, which encodes a subunit of TFIIIC. Perhaps regulated Pol III transcription in this region, or even the binding of solo TFIIIC, affects Pol II transcription of *TFC6*, such that level of this key Pol III transcription factor is controlled by the process of Pol III transcription itself.

***ZOD1* and *ETC* loci might define new classes of genetic elements.** Genes and other genetic elements are defined by a variety of experimental and computational criteria. In many cases, a given genetic element is defined by a single criterion (e.g., an ORF or a mutant phenotype) without knowledge of its biochemical and/or physiological functions. From such definitions of genetic elements, it can be difficult to distinguish between functional elements and evolutionary remnants. Large-scale experimental approaches, such as the one used here, have the potential to identify new classes of functional elements. Although our understanding of *ZOD1* and the *ETC* loci is incomplete, the available information suggests that these may represent new types of functional genetic elements. The *ETC* loci are defined functionally by the presence of TFIIIC, but not other general Pol III factors; thus, they represent "incomplete promoters" where a partial transcription complex is assembled (at least under the conditions tested). *ETC* loci differ from Pol II-specific activator or repressor binding sites, which are bound by proteins that are not part of, and often bind independently of, the general Pol II machinery. It is unclear whether analogous "incomplete Pol II promoters" bound by a subset of general Pol II factors exist in wild-type cells. On the other hand, *ZOD1* behaves as a functional promoter in the sense that it recruits the complete Pol III machinery, but it may encode a nonsensical RNA product. Although the functional properties of *ZOD1* and the *ETC* loci appear to be unusual, their phylogenetic conservation and the preservation of their unusual Pol III recruitment profile in a second yeast species suggests that they play novel physiological roles.

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