

Genome-wide location of yeast RNA polymerase III transcription machinery

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RNA polymerase III (Pol III) transcribes a large set of genes encoding small untranslated RNAs like tRNAs, 5S rRNA, U6 snRNA or RPR1 RNA. To get a global view of class III (Pol III-transcribed) genes, the distribution of essential components of Pol III, TFIIC and TFIIB was mapped across the yeast genome. During active growth, most class III genes and few additional loci were targeted by TFIIC, TFIIB and Pol III, indicating that they were transcriptionally active. *SNR52*, which encodes a snoRNA, was identified as a new class III gene. During the late growth phase, TFIIC remained bound to most class III genes while the recruitment of Pol III and, to a lesser extent, of TFIIB was down regulated. This study fixes a reasonable upper bound to the number of class III genes in yeast and points to a global regulation at the level of Pol III and TFIIB recruitment.

Keywords: ChIP on chip/microarray/transcription/tRNA gene/yeast

Introduction

In eukaryotic cells, three nuclear RNA polymerases (Pol) transcribe distinct classes of genes (Sentenac, 1985). Pol I transcribes a single essential gene, the rDNA, to generate the 35S rRNA precursor. Pol II synthesizes pre-messenger RNAs and a number of small, stable RNA genes involved in mRNA splicing and in the modification of rRNAs. Pol III is essentially responsible for the synthesis of the small RNA species involved in translation like 5S rRNA and tRNAs. In yeast, Pol III was also shown, both *in vivo* and *in vitro*, to synthesize U6 spliceosomal RNA, the 7SL and the RNase P RNAs that are involved in protein secretion and in the maturation of tRNAs, respectively encoded by the *SNR6*, *SCR1* and *RPR1* genes (for a review, see White, 1998). Except for the 5S RNA gene, all known yeast class III genes (i.e. genes transcribed by RNA polymerase III) share control sequence motifs, the A and B blocks, and a run of T residues that specifies transcription termination (Willis, 1993). The transcription start site is located around 20 nucleotides upstream of the A block (see Figure 1 for a model view). In contrast, the B block is

located at a variable distance downstream of the A block within the transcription unit, or exceptionally, downstream of the termination site in the case of the *SNR6* gene. *SNR6* and a few tRNA genes have a TATA-like element upstream of the transcription start site (Brow and Guthrie, 1990; Eschenlauer *et al.*, 1993; Dieci *et al.*, 2000).

In vitro studies with the yeast transcription system have described the cascade of protein–DNA interactions leading to the recruitment of Pol III to its target genes (Chédin *et al.*, 1998; Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002). The first step in the formation of the yeast Pol III transcription initiation complex is the recognition of the A and B blocks by the multisubunit TFIIC factor (see Figure 1 for a model). TFIIC comprises two large protein modules, τ A and τ B (Marzouki *et al.*, 1986). τ B (three subunits) binds to the B block with high affinity and favors A block binding by τ A (three subunits). Elegant protein–DNA crosslinking experiments have provided a coarse mapping of TFIIC subunits over a tRNA gene and the 5S RNA gene (Bartholomew *et al.*, 1990; Braun *et al.*, 1992). The subunit τ 95 used in the present study belongs to the τ A module (Conesa *et al.*, 1993), maps over the A block (Bartholomew *et al.*, 1990) and influences the stability of the TFIIC–DNA complex and start site selection (Jourdain *et al.*, 2003). Once bound, TFIIC directs the assembly of TFIIB upstream of the initiation site (Kassavetis *et al.*, 1990; Bartholomew *et al.*, 1991). TFIIB comprises three components, the ubiquitous TATA binding protein (TBP), Brf1 which is related to TFIIB, and Bdp1. Brf1 appears to play a key role in initiating TFIIB assembly (Kassavetis *et al.*, 1992) and in the subsequent Pol III recruitment and post-recruitment steps (Brun *et al.*, 1997; Kassavetis *et al.*, 1998). Bdp1 locks the TFIIB–DNA complex in a highly stable form competent for the stable recruitment of Pol III over the start site (Kumar *et al.*, 1997). The presence of the TATA box in *SNR6* or some tRNA genes can bypass the requirement for TFIIC *in vitro*. TATA-box directed binding of TFIIB is mediated in that case by TBP (Margottin *et al.*, 1991; Burnol *et al.*, 1993b; Whitehall *et al.*, 1995). Once formed, the TFIIB–DNA complex suffices to recruit the 17 subunit Pol III enzyme (Kassavetis *et al.*, 1990, 1997) via multiple interactions involving specific Pol III subunits (Bartholomew *et al.*, 1993; Werner *et al.*, 1993; Brun *et al.*, 1997; Andrau *et al.*, 1999; Ferri *et al.*, 2000).

In yeast, the 5S RNA genes are arranged in tandem with the 35S rRNA genes. Arrays of 100–200 copies of these genes are present on the right arm of chromosome XII. In contrast, the 275 tRNA genes are dispersed in the genome (Percudani *et al.*, 1997; Hani and Feldmann, 1998). The yeast genome has been studied *in silico* to predict the existence of additional class III genes, based on the

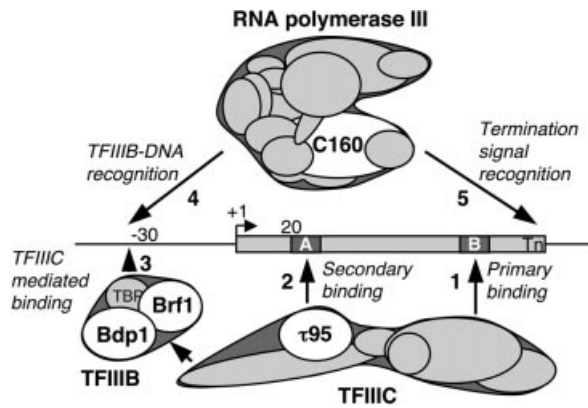


Fig. 1. Transcription complex assembly on a yeast tRNA gene. The scheme depicts the multistep pathway of transcription complex formation: promoter recognition by TFIIC, TFIIC-directed assembly of the initiation factor TFIIB and recruitment of RNA Pol III (numbered arrows) (Chédin *et al.*, 1998; Geiduschek and Kassavetis, 2001). Tagged polypeptide components used in this paper are identified by white ovals.

presence of A and B blocks and a poly-T track within intergenic regions (Olivas *et al.*, 1997). This study identified one additional gene with intragenic A and B blocks, *RNA170*, which indeed showed a dependence on the B block and Brf1 for transcription *in vivo*. Based on the tRNA gene paradigm, this analysis assumed that Pol III transcription would always depend on TFIIC binding, which remains to be established, and relied on a robust conservation of A and B block elements, which is not always the case.

A complete panorama of all the yeast class III genes would be a great asset to potentially reveal new RNA species and to address a number of questions concerning the Pol III promoter elements, the general role of TFIIC, or possible differential regulation of class III genes. In the work presented here, we have used chromatin immunoprecipitation experiments coupled to microarrays hybridization (ChIP on chip; Ren *et al.*, 2000; Iyer *et al.*, 2001; Lieb *et al.*, 2001) to investigate the Pol III genome as defined as the repertory of the binding sites for the Pol III transcription machinery. The binding sites of essential components of Pol III, TFIIC and TFIIB were mapped across the entire yeast genome. All known class III genes and a small set of additional loci were targeted by at least one component of the Pol III transcription machinery.

Results

Specific immunoprecipitation of class III genes with TFIIC

To identify the targets of the Pol III transcription apparatus, we combined chromatin immunoprecipitation and intergenic microarray hybridization. Chromatin proteins were crosslinked with formaldehyde to their target sites *in vivo* (Hecht and Grunstein, 1999; Hecht *et al.*, 1999). After sonication that resulted in chromatin fragments of 250–1000 bp in size, DNA fragments that were specifically crosslinked to the myc-tagged 95 kDa subunit of TFIIC were purified by immunoprecipitation with a monoclonal antibody directed to the epitope tag. To

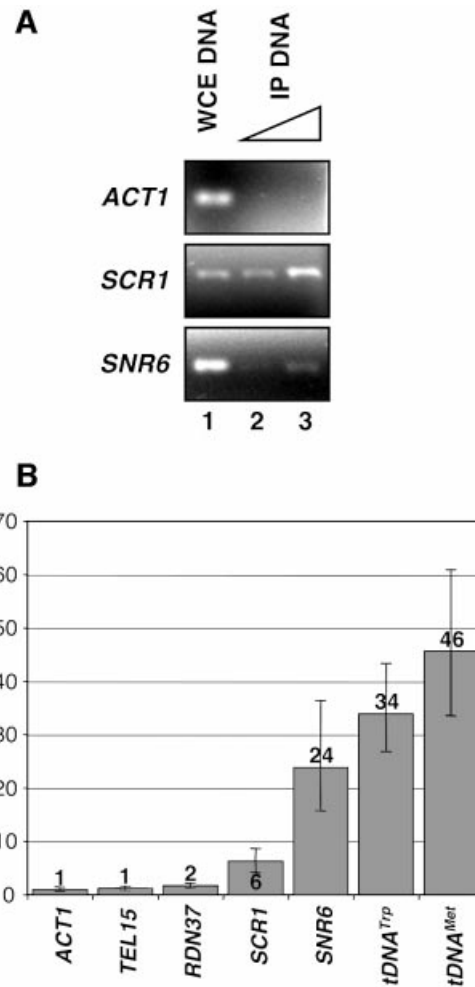


Fig. 2. Gene-specific PCR analysis of immunoprecipitated chromatin bound to $\tau 95$ -myc. (A) Primers hybridizing to the indicated promoter regions were used to amplify DNA immunoprecipitated with anti-myc antibody (lane 2 and 3) from the $\tau 95$ -myc-tagged strain. DNA purified from whole-cell extract (WCE) was used as positive control (lane 1). As a Pol II transcribed gene, *ACT1* is not expected to be enriched in $\tau 95$ -associated chromatin, while the class III genes *SCR1* and *SNR6* contain TFIIC binding sites. (B) Relative enrichment of DNA fragments, immunoprecipitated extracts, from the $\tau 95$ -myc-tagged strain relative to genomic DNA from the whole-cell extract as determined by real-time PCR analysis. Enrichments levels for each gene were normalized to the level of the *ACT1* promoter. The data show the mean, maximum and minimum fold enrichment from three independent experiments.

evaluate the selectivity of the immunoprecipitation reactions, the immunopurified DNA fragments were first analyzed by PCR amplification for the enrichment of two known class III genes, *SCR1* and *SNR6*, that encode the RNA component of the 7SL particle and U6 snRNA, respectively (Figure 2A, lanes 2 and 3), relative to the *ACT1* promoter as *ACT1* (encoding actin) is transcribed by RNA polymerase II. As shown in Figure 2, *SNR6* and *SCR1* loci were amplified while *ACT1* was not, under the same conditions (Figure 2A, compare lanes 3 and 2 with lane 1). In a second set of experiments, we quantified the specific fold enrichment of several known class III genes or non-Pol III DNA by real-time PCR quantification. The fold enrichment observed for each DNA locus was normalized to the fold enrichment observed for the

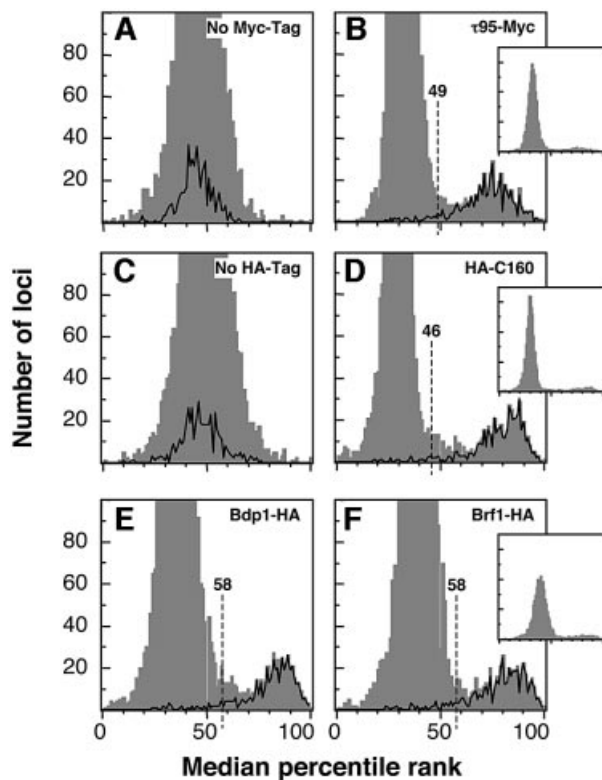


Fig. 3. Specific enrichment of intergenic regions adjacent to class III genes. Distribution of median percentile ranks of Cy3/Cy5 fluorescence ratios are represented as gray histograms. (A) Control immunoprecipitation of untagged protein with an anti-myc antibody or (B) immunoprecipitation of myc-tagged TFIIC. (C) Immunoprecipitation of control untagged protein extract with an anti-HA antibody, (D) tagged HA-C160, (E) tagged Bdp1-HA, and (F) tagged Brf1-HA. Full-scale distributions for (B), (D) and (F) are represented in small insets (maximum y-axis is 700). The distributions of median percentile ranks of 489 flanking class III genes regions (corresponding to 271 tRNA genes, *RPR1*, *SNR6*, *SCR1*) are represented as black curves. The thresholds chosen to identify class III genes are indicated by dotted lines.

ACT1 promoter. As expected, a fragment of chromosome XV telomere (*TEL15*) and a fragment of large rRNA 35S gene (named *RDN37* in the *Saccharomyces cerevisiae* database) did not co-immunoprecipitate with epitope-tagged $\tau 95$ (Figure 2B, enrichment of 1 and 2 for *TEL15* and *RDN37*, respectively). In contrast, the class III promoters of *SCR1*, *SNR6*, *tDNA^{Trp}* [also named tW(CCA)G1] and *tDNA^{Met}* gene [tM(CAU)D] were specifically enriched, from 6- to 46-fold, in immunoprecipitated DNA as compared with the *ACT1* promoter region (Figure 2B). The specific crosslinking of tagged $\tau 95$ to class III genes encouraged us to undertake a genome-wide analysis of TFIIC binding sites.

After reversal of the protein–DNA crosslinks, DNA fragments enriched by immunoprecipitation were amplified and fluorescently labeled with the Cy3 fluorophore. In parallel, a control DNA from whole-cell extract was amplified and fluorescently labeled with the Cy5 fluorophore. Genomic target loci were identified by comparative hybridization of the immunoprecipitated versus the control DNA probes to a DNA microarray harboring intergenic DNA regions. The ratio of the Cy3 to Cy5

fluorescence intensities at each site in the microarray provided a measure of the extent of binding of the bait protein to the corresponding genomic locus (for an example see Figure 4B). We used microarrays of intergenic regions to uncover all potential TFIIC binding sites and to circumvent the problem of cross-hybridizations between conserved sequence elements (such as tRNA and Ty elements). The DNA microarray represented 6402 intergenic intervals on either side of every open reading frame, tRNA, small nuclear RNA, Ty element, solo δ , etc (see Supplementary data, available at *The EMBO Journal* Online). The length of the sheared crosslinked DNA (between 250 and 1000 bp; Supplementary data), the absence of sequence similarity between intergenic elements adjacent to class III genes and the small size of class III genes (the largest class III gene, *SCR1*, is 522 bp long; Dieci *et al.*, 2002) was expected to allow the identification of Pol III promoters by hybridization of the crosslinked DNA fragments to intergenic elements located immediately upstream or downstream of a class III genes.

To identify the targets of the myc-tagged TFIIC, we ranked genomic loci according to their logarithmic fluorescence ratios. The median of the percentile rank values for each locus was determined from four independent experiments. The distribution of the median percentile ranks for the control immunoprecipitation experiment with anti-myc antibodies on wild-type (untagged) cell extracts was Gaussian and thus showed no enrichment of particular genomic DNA fragments (Figure 3A, gray histogram). In contrast, the distribution of the median percentile ranks for the $\tau 95$ immunoprecipitation experiment was bimodal (Figure 3B, gray histogram), which indicates the specific enrichment of a significant number of intergenic elements.

Remarkably, the distribution of 489 class III gene flanking regions (271 tRNA genes, *RPR1*, *SNR6*, *SCR1*) exactly fitted the second bulk of the global distribution of the DNA fragments immunoprecipitated with tagged TFIIC (with the notable exception of *RDN5*, which encodes the 5S rRNA) whereas it was normally distributed in the mock immunoprecipitation experiments (the black curves in Figure 3A and B).

Genome-wide binding of the Pol III machinery

The bimodal distribution and the specific enrichment of the class III-specific intergenic elements in the DNA sample immunoprecipitated with tagged TFIIC demonstrated the specificity of the analysis and therefore warranted a global study of the Pol III genomic loci. We therefore extended our analysis to three other representative components of the Pol III transcription machinery: the largest subunit of Pol III, C160, and the two components of TFIIB, Brf1 and Bdp1. Each of these protein baits were tagged with HA epitopes. As in the case of $\tau 95$, in at least two independent experiments, each protein bait enriched a collection of DNA fragments that fitted well with the known class III genes (see the bimodal distribution in Figure 3D–F), whereas no enrichment of known class III genes was observed in the mock experiment (no HA tag, Figure 3C). We defined for each experiment a threshold percentile rank above which intergenic elements formed a group that was distinct from the bulk of the distribution.

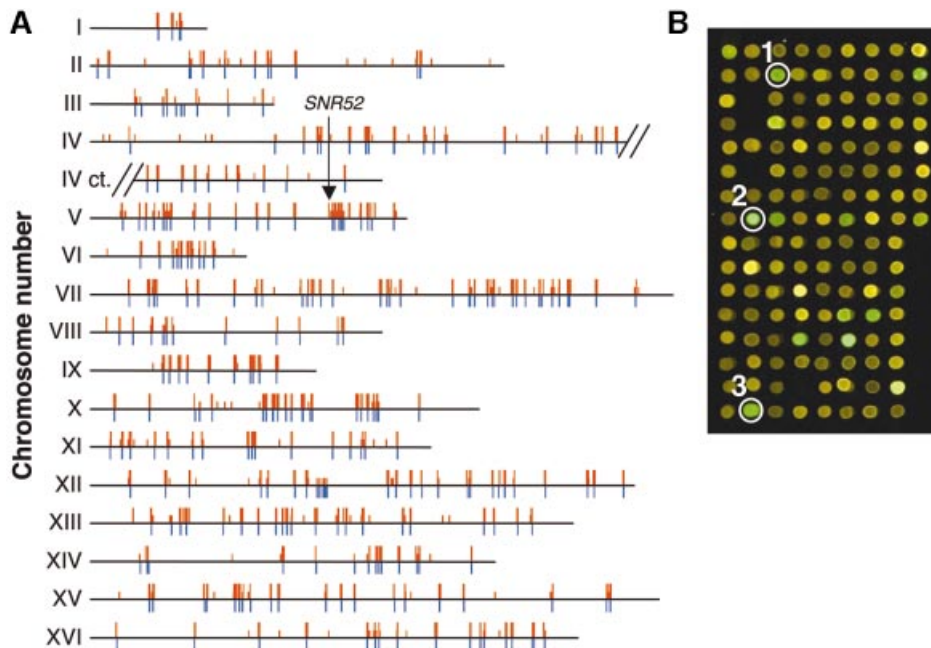


Fig. 4. Chromosomal display of genomic binding sites for $\tau 95$, Bdp1, Brf1 and the 160 kDa subunit of RNA polymerase III. (A) Map of the genomic binding sites of $\tau 95$, Brf1, Bdp1 and C160 determined by CHIP on chip analysis. Red bars correspond to the 647 intergenic regions bound by at least one of the class III transcription machinery, among which the 458 enriched by the four components together are highlighted by higher red bars. The blue bars indicate the position of the 275 annotated tRNA genes and other class III genes *SNR6*, *RPR1*, *SCR1* and *RDN5*. An arrow points to the *SNR52* gene. (B) Partial view of a microarray slide of a $\tau 95$ -myc immunoprecipitation experiment. Spots corresponding to the intergenic region flanking two TATA-containing tRNA genes tP(UGG)F and t(UAU)D (white circles 1 and 2, respectively) and a TATA-less tRNA gene tS(AGA)E (white circle 3) are highlighted. Green spots correspond to enriched intergenic regions.

Slightly different threshold percentile ranks (58th, 58th, 49th and 46th) have been selected for Bdp1, Brf1, $\tau 95$ and C160, respectively (Figure 3B and D–F), due to the better efficiency of the immunoprecipitation reactions with $\tau 95$ and C160 bait proteins. We found 647 loci with a percentile rank above the threshold value with at least one of the four protein baits. Mapping these 647 loci across the whole genome (Figure 4A, red bars) shows a striking colocalization with the known class III genes (Figure 4A, blue bars). Four hundred and fifty-eight loci were selectively enriched with each of the four protein baits (Figure 4A, highest red bars), which corresponded to 7.21% of the 6402 intergenic regions. These 458 loci were assumed to be intergenic elements adjacent to known or unknown class III genes.

These 458 loci were designated LEPTM for loci enriched by the Pol III transcription machinery. Four hundred and forty-two LEPTM (97% of the 458 LEPTM) were located next to or at a close distance from a tRNA gene (Table I), which underscored the quality of the microarrays. Ninety-four per cent of these 442 LEPTM are adjacent to tRNA genes. The size range of the DNA fragments generated by sonication (between 250 and 1000 bp, as estimated by gel electrophoresis) can explain some rare cases of hybridization with loci located farther, 1000–1500 bp, from a tRNA gene. When a locus lay between a pair of class III genes, we considered both genes as potential transcriptional targets. Therefore, the 442 LEPTM characterized the occupancy of the four bait proteins on 259 of the 275 tRNA genes (Table I). On the 16 remaining LEPTM, two were adjacent to the *SNR6* genes, one flanked the *RPR1* gene and another one flanked the

SCR1 gene (Table I). The impressive colocalization of the intergenic elements enriched with four components of the class III transcription apparatus with nearly all the known class III genes (259 of the 275 tRNA genes, plus *SNR6*, *RPR1* and *SCR1*) strongly suggested that the remaining unassigned loci could correspond to unknown class III genes. Twelve LEPTM could not be assigned to any known class III gene and potentially encompassed, or were adjacent to, some unknown class III genes. Among these 12 LEPTM, one (distant from any known class III genes) was adjacent to the *SNR52* gene that encodes the small snoRNA snR52, a methylation guide for rRNA. Six LEPTM mapped near Ty transposons, usually inserted upstream of tRNA genes (Hani and Feldmann, 1998). We found by PCR analysis that three of these Ty elements were deleted in our yeast strains (data not shown) and the corresponding loci were therefore located next to a tRNA gene. Sequence analysis of the remaining three loci upstream of authentic Ty insertions revealed partial sequence identities with other loci adjacent to tRNA genes (data not shown), which might have caused false positives by cross-hybridization. Preliminary northern blot analyses of the last five loci did not reveal the presence of RNA originating from these intergenic elements (data not shown). These negative results were surprising. However, even if one assumes that these loci were false positives, the global level of false positives (<0.1%) was remarkably low due to the stringent criteria of specific enrichment by the four protein baits. Interestingly, these criteria revealed one strong candidate class III gene, *SNR52*.

One could predict, however, that not all class III genes would be enriched by each of the four baits. For instance,

Table I. Summary of the 647 enriched loci

	C160	TFIIIC95	BRF1	BDP1	Number of intergenic regions	Pol III genes
<i>tRNA loci</i>	+	+	+	+	442	259
	+	+	+	+	11	2
	+	+	+	+	11	2
	+	-	+	+	17	2
	+	-	+	+	14	1
	+	-	+	-	5	3
	+	+	-	-	12	2
	+	-	+	+	20	1
	-	+	+	+	1	1
	-	+	-	-	4	1
	-	-	+	+	4	1
	-	-	-	+	6	1
<i>SNR6</i>	+	+	+	+	2	1
	+	-	-	+	1	1
<i>RPR1</i>	+	+	+	+	1	1
	+	-	-	-	1	1
<i>SCR1</i>	+	+	+	+	1	1
<i>RDN5</i>	+	-	-	+	2	2
<i>RNA170</i>	+	+	-	-	1	1
<i>Other loci</i>	+	+	+	+	12	
	+	+	-	+	2	
	+	-	+	+	1	
	+	-	-	+	1	
	+	+	+	-	2	
	+	+	-	-	3	
	+	-	-	-	11	
	-	+	+	+	1	
	-	+	-	-	20	
	-	-	+	+	1	
	-	-	+	+	2	
	-	-	-	+	26	
	-	-	+	-	9	
Total					647	279

the 5S RNA gene was not enriched by $\tau 95$, as mentioned above. Therefore, we further analyzed the 189 loci that were enriched by at least one of the four protein baits. Some of the 189 loci that had median percentile ranks above the threshold in one, two or three of the four immunoprecipitations could be assigned to known class III genes. Hence, 105 loci mapped within 1500 bp from a tRNA gene, adding 14 additional tRNA genes to the 259 tRNA genes already identified with the four bait proteins. In many instances, the enriched loci were not adjacent to the tRNA genes, which explains their selection by a subset of the protein baits and their low percentile rank. Note that the tD(GUC)N gene, which is considered as a pseudo gene due to a Ty insertion immediately upstream of the A box, was immunoprecipitated with $\tau 95$ only. Two loci close to two tRNA genes, at a distance of <1.3 kb on the right arm of chromosome III, were not enriched by any of the protein bait. However, we did not succeed in amplifying this

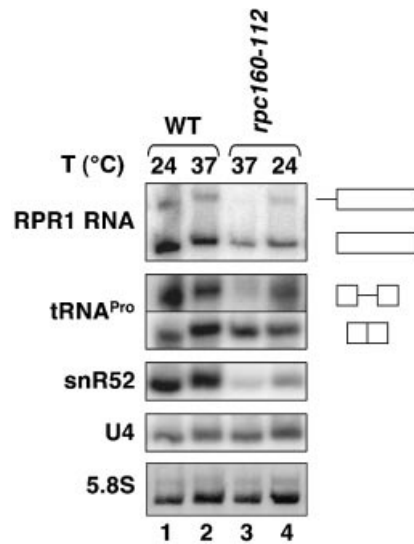


Fig. 5. Northern blot analysis of snR52 from wild-type and *rpc160-112* cells. Wild-type (lanes 1 and 2) or *rpc160-112* (lanes 3 and 4) cells were grown at 24°C (lanes 1 and 4) or shifted for 3 h at 37°C (lanes 2 and 3). RNA extraction, gel electrophoresis, electrophoretic transfer on nylon membrane, and hybridization with DNA probe was carried out as described in Materials and methods. The RNAs probes are indicated on the left. The precursor and mature RPR1 RNA, the 5'- and 3'-processed, unspliced precursor and mature tRNA^{Pro} are indicated on the right. As a loading control, the bottom panel shows a post-transfer methylene blue staining of 5.8S RNA on the membrane.

region by PCR analysis on S288c and YPH500 strain genomic DNA (data not shown). With these two exceptions, all the tRNA genes were targeted by at least one of the protein baits. Five loci flanked or harbored other known class III genes: *RDN5* (two loci), *SNR6* (one locus), *RPR1* (one locus), and *RNA170* (one locus). The remaining 78 loci, which could not be assigned to known class III genes (Table I), also had a relatively low median percentile rank as compared with the class III genes related loci recognized by the four protein baits (data not shown).

SNR52 is transcribed by RNA polymerase III

We have focused our study on the intergenic region adjacent to the *SNR52* gene (Figure 4). *SNR52* has been previously identified by a computational screen as a snoRNA gene required for 2'-O-methylation of rRNA (Lowe and Eddy, 1999). To confirm that the *SNR52* gene was transcribed by Pol III *in vivo*, we analyzed the transcription profile of this gene by northern blot analysis in a mutant strain harboring a thermosensitive mutation *rpc160-112* in the catalytic site of the Pol III largest subunit (Dieci et al., 1995). Total RNA was extracted from wild-type and mutant cells grown at permissive temperature (24°C) or 3 h after a temperature shift at 37°C, fractionated by electrophoresis, transferred onto a membrane and hybridized with various probes (Figure 5). While the level of control spliceosomal U4 RNA synthesized by RNA polymerase II was unaffected by the temperature shift (Figure 5, lanes 1–4), that of 5'- and 3'-processed but unspliced precursor of tRNA^{Pro} was drastically decreased in mutant cells, as expected for a Pol III transcript (Figure 5, compare lanes 2 and 3, upper

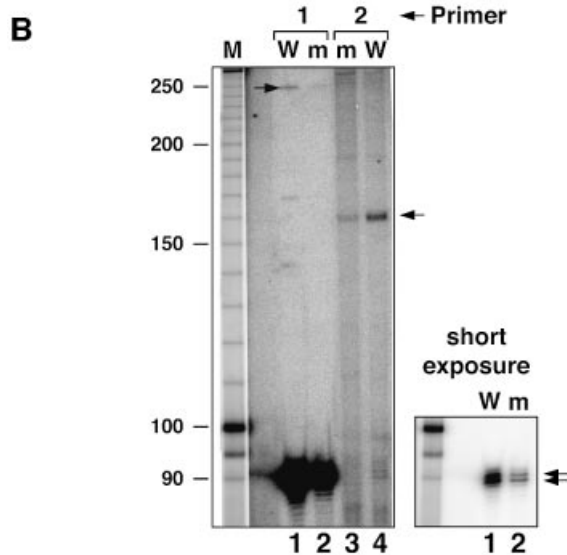
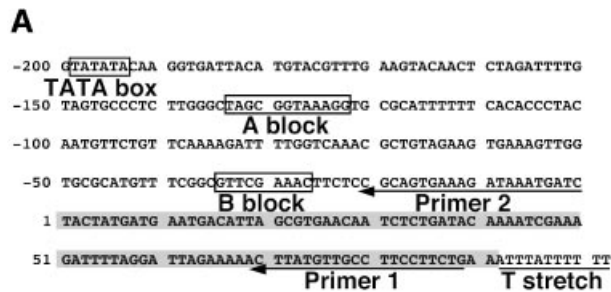


Fig. 6. *SNR52* gene and RNA transcripts. (A) *SNR52* gene sequence shows canonical class III promoter elements (boxed) in a leader sequence and a putative Pol III termination signal (underlined T stretch). The sequence of mature snR52 is labeled in gray (Lowe and Eddy, 1999); arrows underline the sequence of Primers 1 and 2 used for primer extension. (B) Primer extension analysis of *SNR52* snoRNA and its precursor. Total RNA (3 μ g) from YPH500 (W; lanes 1 and 4) or *rpc160-112* mutant cells (m; lanes 2 and 3) grown at 24°C was reverse transcribed using Primer 1 (lanes 1 and 2) or Primer 2 (lanes 3 and 4).

panel). The transcription defect was not apparent at the level of mature tRNA^{Pro} due to the stability of tRNAs (Figure 5, compare lanes 2 and 3, lower panel). Similarly, the precursor RPR1 RNA was undetectable and the mature RPR1 RNA level was reduced in the mutant cells grown at 37°C (Figure 5, compare lanes 2 and 3). The level of unspliced and mature RPR1 RNA was reduced even at permissive temperature (24°C) in the mutant cells although the same amount of total RNA was analyzed in these northern blot experiments (Figure 5, compare lanes 1 and 4). Similarly, the level of snR52 was reduced in the *rpc160-112* cells grown at permissive temperature (Figure 5, compare lanes 1 and 4) and became nearly undetectable at the restrictive temperature (Figure 5, lane 3).

Interestingly, two transcripts of ~90 (Figure 5) and ~250 (data not shown) nucleotides were revealed by this northern blot analysis of the *SNR52* gene product. The size of the most-abundant RNA was in good agreement with the predicted size of snR52 (92 bases long; see Figure 5; Lowe and Eddy, 1999). The second RNA band

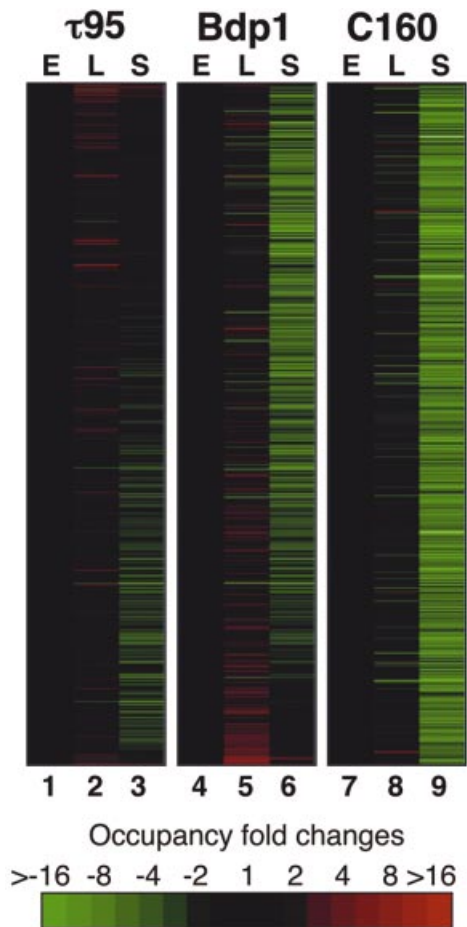


Fig. 7. Occupancy profiles of loci enriched by the Pol III transcription machinery (LEPTM) at different growth phases. Four hundred and nineteen LEPTM arranged in rows are classified according to their enrichment fold changes from exponential (columns E, normalized to 1) to late exponential (L) and stationary (S) growth phases for τ 95, Bdp1 and C160 bait proteins. As indicated on the color scale, black means no changes whereas green and red indicate decreased and increased DNA binding levels, respectively.

was present at a very low level (about 0.5% of the ~90 nucleotide transcript) in the wild-type strain and was undetectable in the mutant strain (data not shown). To investigate whether this longer RNA band was a precursor of snR52, we performed a primer extension analysis using two probes. The first one (Primer 1, located 3 nucleotides from the 3'-end; see Figure 6A) will hybridize with the snR52 RNA (Lowe and Eddy, 1999) and the other one, located just upstream of the predicted mature snR52 5'-end (Primer 2; see Figure 6A), should hybridize with a putative precursor. Two DNA fragments of 89 and 90 bases long were observed with the 3' probe and therefore corresponded to the predicted snR52 sequence (Lowe and Eddy, 1999). A second DNA fragment of ~250 bases, 160 bases longer than the predicted sequence, was synthesized with the same primer (Figure 6B). PhosphorImager analysis indicated that this large fragment was ~1000-fold less abundant than the 89–90 base fragments. The 90 and 250 base DNA fragments corresponded well to the small and large snR52 transcripts observed by northern blot. Finally, a DNA fragment of 160 bases was synthesized by

extension of the second primer located just upstream of the predicted sequence (Figure 6B, lanes 3 and 4). These results suggested that snR52 is synthesized as a precursor RNA with a leader sequence of 160 bases that is efficiently cleaved to generate the 92–93 base mature snR52. We therefore looked for promoter elements in this putative leader sequence. Indeed, sequence analysis revealed a well-conserved A block element (one mismatch to the tRNA gene consensus) ~26 bp downstream of the predicted 5'-end of the transcript, a perfect B block 87 bp downstream, and a TATA-like element ~33 bp upstream of the predicted start region (see Figure 6A). In addition, as expected, the snR52 gene ends with a stretch of T residues, typical for Pol III termination sequences. Furthermore, a Ty1 LTR scar of a past transposition event is present about 110 bp upstream of the predicted start region, in good agreement with the known colocalization of retrotransposons with class III genes (Hani and Feldmann, 1998; Sandmeyer, 1998).

Down regulation of Pol III and TFIIB recruitment in stationary-phase cultures

Yeast Pol III transcription decreases markedly during late growth phase (Sethy *et al.*, 1995; Clarke *et al.*, 1996). We analyzed the occupancy of class III genes by τ 95, Bdp1 and C160 during exponential, late-exponential and stationary growth phases to investigate on a genomic scale the basis of this regulation and whether all the genes would be regulated in a coordinated manner (see Materials and methods). To compare the three growth stages, we determined for each of 419 LEPTM the fold enrichment of the late-exponential and stationary phase relative to the exponential phase (Figure 7). The data were subjected to hierarchical clustering and visualized with TreeView (see Supplementary data). With the C160 bait, the 419 LEPTM were found to be uniformly and markedly less enriched during stationary phase relative to the exponential phase (Figure 7, compare lanes 9 with lanes 3 and 6). This low occupancy of class III genes by RNA polymerase III in stationary phase is in keeping with the general decrease in Pol III transcription. Most of the 419 LEPTM were also significantly less enriched in stationary phase with the Bdp1 bait. However, the decrease of Bdp1 binding was less pronounced than with the C160 bait (Figure 7, compare lane 6 with lane 9). Notably, some intergenic elements (59 of the 419 LEPTM analyzed) were equally enriched in stationary and exponential-phase cells. Most remarkably, with the τ 95 bait, the LEPTM were either equally enriched in stationary and exponential phases (249 LEPTM) or their enrichment was much-less affected in stationary phase than in the case of C160 or Bdp1 (Figure 7, compare lane 3 with lanes 9 and 6). We noted that an intergenic element adjacent to *RPR1* was one of eight loci that were equally occupied in exponential and stationary phases by the two transcription factors TFIIC and TFIIB, whereas Pol III binding was much decreased; there were also cases (51 LEPTM) where TFIIC (τ 95) binding decreased, whereas TFIIB (Bdp1) binding did not (Figure 7, lower part). These results suggest that, in stationary-phase cells, Pol III transcription is globally regulated at the level of TFIIB and Pol III recruitment while TFIIC remains mostly bound to the genes.

Discussion

We have mapped the genomic binding sites for four components of the Pol III transcription apparatus which are each essential for TFIIC, TFIIB or Pol III activity. Based on the enrichment of intergenic regions co-immunopurified with epitope-tagged proteins, the colocalization of the four proteins targeted 94% of the tRNA genes and identified a new class III gene, *SNR52*. All the known class III genes and a relatively small set of additional loci were targeted by at least one component of the Pol III transcription machinery, which suggests an upper bound to the number of class III genes in *S.cerevisiae*.

The four components selected for this study are the largest subunit of Pol III (C160), two components of TFIIB (Brl1 and Bdp1), and τ 95, a TFIIC subunit involved in A block binding. All four are part of multiprotein complexes and although none was shown to bind DNA by itself *in vitro*, each of them is thought to contact DNA during pre-initiation or transcription complex formation (Huet *et al.*, 1997; Colbert *et al.*, 1998; Persinger *et al.*, 1999; Shah *et al.*, 1999; Geiduschek and Kassavetis, 2001; Juo *et al.*, 2003). Therefore, crosslinking of τ 95 to genomic DNA can be considered as a signature for TFIIC binding, crosslinking of Brl1, which initiates TFIIB assembly, and Bdp1 that locks TFIIB–DNA complex in its highly stable form both witness TFIIB binding, and crosslinking of C160 indicates transcription complex formation or elongation. In the clearest case where the four components colocalized (i.e. enrich the same intergenic regions), we infer that the genes were transcribed by Pol III in a TFIIC- and TFIIB-dependent manner. This was the case for 259 tRNA genes (out of 275) and three additional class III genes *SNR6*, *RPR1* and *SCR1*. It is interesting to note that the vast majority of the redundant tRNA genes were found to be transcriptionally active under active growth conditions (exponential phase in rich medium). Together with the observation that there is a good correlation between tRNA gene redundancy (copy number) and the overall amino acid composition of yeast proteins, this result confirms the assumption that intracellular levels of tRNA in growing yeast cells are mainly determined by gene copy number (Percudani *et al.*, 1997).

Using the same criteria, six other loci targeted by the four components were strong class III gene candidates. Among them is *SNR52*, which encodes an snoRNA, snR52, required for 2'-O-methylation of rRNA (Lowe and Eddy, 1999). The observation that snR52 RNA level was severely reduced in a Pol III mutant provided further evidence that *SNR52* is a class III gene. Sequence analysis revealed the presence of a T-rich termination site at the end of the gene and well conserved A and B blocks upstream of the snR52 sequence. This promoter configuration is reminiscent of that of the *RPR1* gene that encodes the RNA subunit of RNase P (Lee *et al.*, 1991a,b). *RPR1* gene harbors a transcribed leader sequence with internal A and B block elements recognized by TFIIC. Similarly, northern blotting and primer-extension experiments revealed a mature form of snR52 of the expected length (90 nt) and a minor version of ~250 nt that encompasses the putative A and a B block elements. The very low level

of the long RNA species suggests a very efficient maturation process. Despite the presence of tRNA gene-like promoter elements, the 5'-leader regions of *RPR1* and *SNR52* cannot be folded into a cloverleaf structure characteristic of tRNAs, and did not share other sequence motifs. It would be interesting to know whether the precursors of these two RNA species are processed by the same mechanism. As the function of snR52 is to serve as a methylation guide for rRNA (Lowe and Eddy, 1999), the activity of Pol III is therefore important for rRNA modification. This observation is reminiscent of studies that reported some cross-talks between transcription of class III genes and rRNA processing (Hermann-Le Denmat *et al.*, 1994; Dechampesme *et al.*, 1999; Briand *et al.*, 2001).

Northern blot experiments on the five remaining loci targeted by the four protein baits did not reveal any detectable RNA species. These negative results are intriguing. Formally, they do not exclude that these loci recruit the Pol III machinery unproductively, or that the transcripts are much-less abundant than usual class III RNAs and remained undetected. Alternatively, these few loci could be false positives. Decreasing the criteria stringency was nevertheless warranted by the observation that some bona fide class III genes were detectably targeted by only one, two or three of the four components (see Table I). Considering the 189 loci that were enriched with one, two or three protein baits, 105 mapped within 1500 bp from a tRNA gene and therefore were assigned to these genes since they mapped within the limit of resolution of the method. This assignment was reasonably validated by the recovery of 14 missing tRNA genes that were not targeted by the four components together (259 out of 275 tRNA genes). The decision on stringent thresholds was in part responsible for the incomplete recovery of all tRNA genes by the four protein baits but had the advantage of reducing false positives to a minimum. Therefore, one can expect that new class III genes will be identified among the 78 still unassigned loci that were selected by a subset of the transcription components. These 78 loci, which are scattered in the genome, appear to be an upper bound to the number of undiscovered class III genes in yeast. This upper bound may have to be reconsidered since additional intergenic (inter-ORF) regions were recently identified by sequence comparison of close yeast species (Kellis *et al.*, 2003). Considering that the 110/189 ratio of bona fide class III genes among the loci enriched by three or less baits parallels the ratio of seven class III genes among the 12 loci selected by the four components (that include five false positives), one may suspect, however, that the number of new class III genes yet to be discovered will be very limited.

A few tRNA genes did not bind TFIIC detectably, which raises the question of the general requirement for TFIIC in Pol III transcription. Some tRNA genes and *SNR6* have a TATA box that directs TFIIC-independent transcription *in vitro* (Dieci *et al.*, 2000). TFIIB assembly in that case is initiated by TBP binding (Whitehall *et al.*, 1995). However, TFIIC was shown to be required for *SNR6* transcription *in vivo* (Burnol *et al.*, 1993b; Gerlach *et al.*, 1995) and *in vitro* on chromatin templates (Burnol *et al.*, 1993a; Eschenlauer *et al.*, 1993). In the present

experiments, the four tRNA genes with a TATA box (Dieci *et al.*, 2000) were found to be targeted by TFIIC (see Figure 4B). The lack of significant binding of $\tau 95$ to the 5S RNA genes was unexpected since there is also a clear requirement for TFIIC for 5S RNA transcription *in vitro* and *in vivo* (Lefebvre *et al.*, 1994). On 5S RNA gene, the oriented binding of TFIIC is directed by DNA-bound TFIIA. No sequence-specific binding site for TFIIC alone has been detected (Braun *et al.*, 1989; Challice and Segall, 1989). It is possible that TFIIA binding may have reduced $\tau 95$ crosslinking efficiency under the threshold value. Therefore, in yeast, until a counter example is clearly identified, TFIIC appears to be a general transcription factor for class III genes. The above considerations concerning TFIIC apply to Brf1 and Bdp1. There are cases of bona fide class III genes that bound only one of these TFIIB components or none of them (*RDN5* and *RNA170*; Table I). However, these genes are transcribed *in vitro* by a TFIIB-dependent mechanism and the *in vivo* synthesis of RNA170 is affected in a *brf1* mutant strain (Kassavetis *et al.*, 1990; Olivas *et al.*, 1997). The discrepancies illustrate the complexity of interpreting negative results and are likely related to our selection of stringent thresholds. On the other hand, beside a role in transcription, the possibility remains that unproductive binding of some Pol III transcription component may have some local regulatory role. This could well be the case for TFIIC, which can influence the local chromatin state (Marsolier *et al.*, 1995).

The ChIP on chip technique provides a global, though indirect, analysis of the activation and transcription status of all the class III genes, which would be difficult to reach by northern blot or priming extension analysis due to the number of genes and the redundancy of tRNA genes. This technique also allows a more precise evaluation of the *in vivo* DNA occupancy by the different components of the transcription machinery (TFIIB, TFIIC and Pol III) than *in vivo* footprinting. The present analysis revealed that Pol III and, to a lesser level Bdp1, dissociated during stationary phase whereas TFIIC mainly remained bound. Early genome footprinting experiments revealed a near complete Dnase I protection of the upstream regions of the *SUP53* tRNA_{3^{leu}, from -40 to +15 in exponentially growing cells and from -40 to -10 in stationary phase cultures (Huibregtse and Engelke, 1989). Interpreted in terms of TFIIB and Pol III binding (Kassavetis *et al.*, 1990; Bartholomew *et al.*, 1993), these observations suggest the loss of Pol III but the persistence of TFIIB binding in stationary phase. The present analysis confirms and extends the loss of Pol III to all class III genes but suggests instead a global drop of TFIIB binding on the majority of the genes. It is interesting that, while the assembly of Pol III transcription complexes goes stepwise from TFIIC binding to TFIIB assembly then Pol III recruitment, the tightness of the regulation appears to be in reverse order, essentially at the level of Pol III recruitment, less so on TFIIB binding and not detectably on TFIIC binding. It is probably of interest for the cells to maintain a partial level of gene activation with bound TFIIC and some TFIIB binding in order to rapidly resume transcription when growth conditions improve.}

Materials and methods

Yeast strains, growth conditions and construction of epitope-tagged strains

The 3HA-C160 (MW671) strain has been described elsewhere (Dieci *et al.*, 1995). All strains were derived from YPH499 or YPH500 strains (Sikorski and Hieter, 1989). 3 HA- or 13 myc-tagged strains were constructed as described previously Longtine *et al.* (1998). The epitope-coding sequence was inserted between the last codon and the stop codon of the target ORF. The proteins Brf1, Bdp1 and τ 95 were tagged, respectively, by 3 HA-, 3 HA- and 13 myc-epitope (in the MW4034, MW4035 and yOH1 strains, respectively). No growth defect was detected for the Bdp1-HA- and τ 95-myc-tagged strains. The Brf1-HA-tagged strain grew normally at 24°C, but its growth was reduced at 30°C compared with the wild-type strain and stopped at 37°C. Thus, it was grown at 24°C instead of 30°C for the other strains. Expression of HA- and myc-tagged proteins was verified by western blot analysis using the 12CA5 (anti-HA) and 9E10 (anti-myc) mouse monoclonal antibodies (both a gift from the DRIP, CEA/Saclay). The wild-type and *rpc160-112* strains used in northern blot and primer-extension experiments are YPH500 and MW670, respectively (Sikorski and Hieter, 1989; Dieci *et al.*, 1995).

To analyze the binding of the Pol III transcription machinery at different growth phases, cells from the τ 95-myc-, Bdp1-HA- or C160-HA-tagged strains were harvested from YPD medium at \sim 0.7 OD₆₀₀ (exponential phase), \sim 4.8 OD₆₀₀ (late exponential phase) and \sim 10 OD₆₀₀ (stationary phase).

Northern blotting and primer extension

RNA extraction and northern blot analysis were performed as previously described (Burnol *et al.*, 1993b) using body-labeled DNA fragments encompassing the sequence of the probed RNA. Quantifications were performed using PhosphorImager apparatus and software (Amersham Pharmacia).

Primer extension was performed using 3 μ g of RNA as described previously (Prioleau *et al.*, 1994). Yeast cells (25 ml cultures) were grown in YPD medium at 30°C to an OD of \sim 0.7. After two washes with sterile water, total yeast RNA was purified (Burnol *et al.*, 1993b) and primer extensions were performed with oligonucleotide primers specific for the snR52 mature sequence or the putative leader sequence of snR52. The sequences of the oligonucleotides used are as follows: Primer 1, 5'-CAGAAGGAAGGCAACATAAGT-3'; Primer 2: 5'-GATCATTATC-TTCTACTGCGG-3'.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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