Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 9338–9342, September 1995
Genetics

The only essential function of TFIIIA in yeast is the transcription of 5S rRNA genes

(RNA polymerase III/*RPR1* gene)

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Communicated by Donald D. Brown, Carnegie Institution of Washington, Baltimore, MD, July 7, 1995

ABSTRACT **We have developed a system to transcribe the yeast 5S rRNA gene in the absence of the transcription factor TFIIIA. A long transcript was synthesized both *in vitro* and *in vivo* from a hybrid gene in which the tRNA-like promoter sequence of the *RPR1* gene was fused to the yeast 5S RNA gene. No internal initiation directed by the endogenous 5S rDNA promoter or any processing of the hybrid transcript was observed *in vitro*. Yeast cells devoid of transcription factor TFIIIA, which, therefore, could not synthesize any 5S rRNA from the endogenous chromosomal copies of 5S rDNA, could survive if they carried the hybrid RPR1–5S construct on a multicopy plasmid. In this case, the only source of 5S rRNA was the precursor RPR1–5S transcript that gave rise to two RNA species slightly larger than wild-type 5S rRNA. This establishes that the only essential function of TFIIIA is to promote the synthesis of 5S rRNA. However, cells devoid of TFIIIA and surviving with these two RNAs grew more slowly at 30°C compared with wild-type cells and were thermosensitive at 37°C.**

In eukaryotes, 5S ribosomal RNA synthesis by RNA polymerase III (Pol III) requires the presence of three transcription factors, TFIIIA, -B, and -C (reviewed in ref. 1). TFIIIA is specific for 5S rRNA genes and responsible for the first step in transcription: the binding to the internal control region (ICR) of the gene. The binding of TFIIIC follows, then TFIIIB, the Pol III initiation factor, is recruited.

TFIIIA is one of the most extensively studied and, hence, characterized transcription factors, for several reasons. First, it has the striking property of binding both 5S DNA and 5S RNA. Furthermore, its study led to the discovery of the zinc-finger motifs, which are structural units found only in eukaryotes and which are responsible for DNA binding (2, 3). These properties, common to all TFIIIA proteins studied so far, were first identified in *Xenopus* TFIIIA, whose study was facilitated by its presence in large amount in oocytes. *Xenopus* TFIIIA possesses nine zinc fingers with specialized functions. The three amino-terminal fingers are primarily responsible for DNA binding, whereas fingers 4–7 are involved in RNA binding. The three carboxyl-terminal fingers and a 14-amino acid sequence in the carboxyl region of TFIIIA are required for transcriptional activation (4–6). Multiple activities have been attributed to *Xenopus* TFIIIA, including a role in DNA relaxation, double-stranded DNA reassociation (7, 8), and being a weak DNA-dependent ATPase (9). These activities could be directly relevant to the mechanism of gene activation. TFIIIA could also have a regulatory role. In *Xenopus, in vitro* transcription complexes preassembled on 5S genes can prevent nucleosomal repression (10). *In vivo*, the relative equilibrium between the amount of TFIIIA and histone H1 is, at least for oocyte 5S genes, very likely a major determinant of the activation (11, 12). Recently, it was also shown in a human homologous *in vitro* system that binding of TFIIIA could prevent nucleosomal repression (13). TFIIIA can also exert a negative-feedback regulation through its binding to 5S RNA. Indeed, it was shown that 5S rRNA inhibits its own synthesis by sequestering TFIIIA (14–16). Finally, besides its role in transcription, *Xenopus* TFIIIA was shown to be involved in the nucleocytoplasmic transport of 5S rRNA and to form cytoplasmic storage 7S ribonucleoprotein particles with 5S rRNA in oocytes (16, 17).

In yeast, less information is available on TFIIIA. Two polypeptides of approximately 50 kDa copurify with TFIIIA activity and can be photocrosslinked to a 5S gene (18–20). Both polypeptides are transcriptionally active *in vitro*. The *TFC2* gene, encoding yeast TFIIIA, has been isolated (21, 22). It is an essential gene and codes for a protein with nine zinc fingers but with only 20% sequence identity to *Xenopus laevis* TFIIIA. Deletion studies of TFIIIA have shown that the three amino-terminal fingers are responsible for both the binding to the box C internal promoter sequence and the recruitment of TFIIIC. Transcription activation requires the presence of an additional 81-amino acid sequence between fingers 8 and 9 of TFIIIA (19). The role of yeast TFIIIA in processes other than 5S transcription has not yet been explored. The present report establishes that if such additional functions exist, they are dispensable for yeast cell viability. We have developed a chimeric 5S RNA gene which allows the synthesis *in vivo* of 5S RNA in the absence of TFIIIA and show that yeast cells expressing this gene survive in the absence of an active TFIIIA gene.

MATERIALS AND METHODS

Media, Plasmids, and Strains. Yeast genetic techniques and media were as described by Ausubel *et al.* (23). Yeast transformation was done according to the lithium acetate procedure. Plasmid pJA230 is a centromere-containing plasmid with a 10-kb insert of yeast DNA spanning the *RPO26* and *TFC2* genes and *URA3* as the selectable marker (24). Plasmid pRS-RPR1-5S is derived from the multicopy plasmid pRS424-5′3′RPR described by Pagan-Ramos *et al.* (25), which contains the 5′ and 3′ sequences flanking the mature domain of the *RPR1* gene, together with *TRP1* as the selectable marker. pRS424-5′3′RPR was linearized by digestion with *Eco*RI, which cuts at the end of the 84-bp *RPR1* leader promoter sequence, blunt-ended by digestion with mung bean nuclease, dephosphorylated, and ligated with a DNA fragment containing the yeast 5S DNA sequence, which was amplified by PCR from the pBS-5S plasmid. The primer at the 5′ end of 5S DNA used for the amplification was 18 nt long and began at nt +2 (the second G) of the 5S DNA. The sequence at the junction between *RPR1* and 5S DNA is GATTGGCAG*GTTGCG; the G* represents G + 1 of the 5S DNA, and the underlined sequence corresponds to the upstream part of the 5′ primer.

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Abbreviations: ICR, internal control region; Pol III, RNA polymerase III; 5-FOA, 5-fluoroorotic acid.

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The primer at the 3′ end was 32 nt long, and it encoded a row of T residues corresponding to a Pol III terminator site and a *Bam*HI restriction site. Another version of 5S DNA was amplified which contained a 25-bp sequence inserted at the unique *Bst*EII site in the ICR of the 5S rDNA. A similar RPR1–5S construct was also generated in a centromere-based plasmid (pRS314-5′3′RPR; a gift from D. Engelke, University of Michigan, Ann Arbor). Plasmid pBS-5S is a Bluescript SK-based plasmid containing a 450-bp *Eco*RI–*Hin*dIII fragment carrying a yeast 5S rDNA sequence. The genotype of the yeast strain YRW1 is MATa *can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 ade2-1 tfc2::LEU2.* The strain is derived from the LP112 strain described in Archambault *et al.* (24). It has the entire coding region of the *TFC2* gene deleted and replaced by a *LEU2* fragment (R. Willis and J. Segall, personal communication) and survives with the *TFC2* gene carried on plasmid pJA230. YSC14 is identical to YRW1, except that plasmid pJA230 is replaced by the multicopy plasmid pRS-RPR1-5S.

***In Vitro* Transcription Assays.** 5S DNA transcription was assayed as described in Lefebvre *et al.* (26) in the presence of *in vitro* translated TFIIIA, affinity-purified TFIIIC, partially purified TFIIIB, and purified Pol III. Final $MgCl_2$ concentration was 12 mM when assaying 5S DNA-type transcription in the presence of TFIIIA and 5 mM when assaying tDNA-type transcription in the absence of TFIIIA. *In vitro* synthesis of TFIIIA was performed as described in Archambault *et al.* (21).

RNA Extraction and Northern Analysis. Small RNA was extracted as described in Hermann-Le Denmat *et al.* (27) from 20-ml yeast cultures with hot phenol, separated on a denaturing 6% polyacrylamide gel, and visualized by ethidium bromide staining. For Northern analysis, RNA was electrophoretically transferred to a positively charged nylon membrane (Boehringer Mannheim) at 400 mA for 1 h in 50 mM Tris-borate EDTA buffer (TBE, 1× TBE = 90 mM Tris-borate/3 mM EDTA) and UV-crosslinked to the membrane in a UV Stratalinker apparatus. Hybridization was performed according to Church and Gilbert (28) in a buffer containing 0.5 M $NaHPO_4$, pH 7.2/10 mM EDTA/7% SDS at 65°C overnight with a ^{32}P-labeled oligonucleotide complementary to the insert introduced in the ICR of the 5S rDNA. The filter was washed four to six times in 40 mM $NaHPO_4$, pH 7.2/1% SDS at 65°C, dried, and autoradiographed at −70°C.

RESULTS

Transcription of a 5S rRNA Gene Under the Control of the *RPR1* Promoter Sequence. A chimeric gene was constructed in which the first 84 nt of the *RPR1* gene containing tRNA-like promoter sequences equivalent to the A and B boxes was fused to a 5S rRNA gene containing its normal termination site (a run of T residues) (Fig. 1). This construct contains the internal promoter element of the 5S rRNA gene, extending from nt +56 to +94 as defined recently by *in vivo* studies (29). It also contains part of the 5S start site element (sse) from +1 to +8, which was shown to be important in *in vitro* transcription studies (30). To assay the function of these various promoter sequences, the RPR1–5S DNA was transcribed in an *in vitro* Pol III-reconstituted transcription system (Fig. 2*A*). Two types of hybrid genes were transcribed: the RPR1–5S DNA construct described in Fig. 1 (Fig. 2*A*, lanes 2 and 3) and the hybrid construct containing a 25-bp sequence in the ICR of the 5S DNA (RPR1–5S maxigene, lanes 4 and 5). In the absence of TFIIIA, a single RNA band was observed in both cases, either 205 nt long for the first construct (Fig. 2*A*, lane 2) or 230 nt for the second (Fig. 2*A*, lane 4). Both had the expected size for RNA transcripts initiated at the *RPR1* promoter sequences and terminated at the T-rich termination site of the 5S rRNA gene. The addition of TFIIIA in the transcription reaction slightly increased the amount of transcript synthesized, probably due to the large amount of unspecific proteins added, but did not change the size of the transcript (Fig. 2*A*, lanes 3 and 5). No RNA originating from the normal 5S initiation site was detected. We conclude that the *RPR1* promoter sequence is functional and dominant over the downstream 5S rDNA promoter sequences.

In yeast, the *RPR1* RNA is made as a precursor that is processed to give rise to the mature *RPR1* RNA (31). One of the processing events corresponds to the removal of the 84-nt promoter region. In the transcription experiment described above, no processing of the RNA to smaller forms was detected. The incubation of the 205-nt-long RPR1–5S precursor RNA with whole yeast extracts did not give rise to smaller RNA species (data not shown). Hence, either the processing enzyme(s) was inactivated in the extracts or the sequence requirements for the processing were not fulfilled.

***In Vivo* Expression of the RPR1–5S Hybrid Construct.** The expression in yeast of the RPR1–5S hybrid gene carried on a multicopy plasmid was followed by Northern blot analysis (Fig. 2*B*). To distinguish 5S rRNA molecules originating from the RPR1–5S RNA gene construct from the endogenous 5S

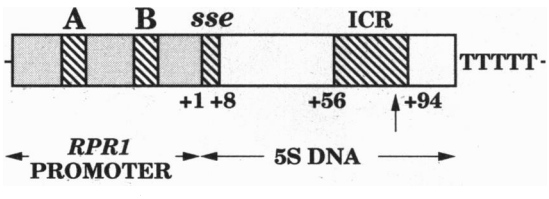

FIG. 1. RPR1–5S construct. A hybrid gene was constructed between the first 84 bp of the *RPR1* gene and the 121 bp of the 5S rRNA gene. The shaded rectangles represent *RPR1* sequences, the open rectangles represent 5S rDNA sequences, and the hatched boxes show the location of the promoter elements: the A and B boxes, the start site element (*sse*), and the ICR of the 5S rDNA. The sequence of T residues shows the location of the Pol III terminator sequence. The numbering refers to the 5S rDNA sequence. The arrow points to the location of the 25-bp sequence added in the maxigene.

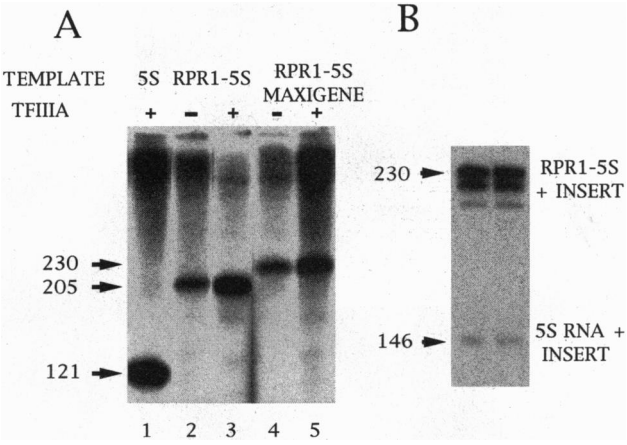

FIG. 2. *In vitro* transcription and *in vivo* expression of the RPR1–5S constructs. (*A*) *In vitro* transcription reactions were performed as described in *Materials and Methods* in the absence of TFIIIA or in the presence of 2 μl of *in vitro* synthesized TFIIIA as indicated. Three types of genes were tested: the 5S RNA gene (lane 1), the RPR1–5S hybrid gene (lanes 2 and 3), and the RPR1–5S maxigene containing a 25-bp insert in the ICR of the 5S DNA (lanes 4 and 5). (*B*) Yeast YRW1 cells were transformed with the plasmid containing the hybrid RPR1–5S maxigene with a 25-bp insert in the ICR. Small RNAs were extracted, separated by electrophoresis on a denaturing polyacrylamide gel, and hybridized with a radioactive oligonucleotide probe complementary to the insert. The membrane was autoradiographed overnight with an intensifying screen at −70°C. The two lanes are identical and correspond to 10 μg of total RNA. The positions expected for the precursor RPR1-5S + insert and for the mature 5S RNA + insert are shown; they correspond respectively to 230 and 146 nt.

rRNA, a sequence of 25 bp was inserted in the ICR of the hybrid gene, and RNA transcribed from the construct was detected by hybridization to a probe complementary to the insert. A major RNA band of 230 nt was detected, corresponding to the size of the precursor RNA synthesized *in vitro* (Fig. 2*A*, lanes 4 and 5). The RPR1–5S hybrid gene could therefore be transcribed *in vivo*. Shorter RNA species were detected, including a minor RNA band of 146 nt corresponding to the expected size for 5S rRNA containing the small insert. This RNA could either originate from the larger transcript by processing or correspond to an initiation at the usual start site in 5S rDNA.

The RPR1–5S Hybrid Gene on a Multicopy Plasmid Can Complement a *tfc2* Deletion at 30°C. Although 5S RNA synthesized from the RPR1–5S construct appeared primarily in the unprocessed form, we tested the capacity of this construct to restore growth of cells devoid of endogenous 5S rRNA from the lack of TFIIIA. A haploid yeast strain (YRW1) carrying a complete deletion of the chromosomal *TFC2* gene coding for TFIIIA and a wild-type *TFC2* copy on a centromeric plasmid was transformed with one of the following RPR1–5S DNA constructs: an RPR1–5S hybrid gene on a multicopy plasmid, the same gene on a centromeric plasmid, or the maxigene with the small insert in the ICR on a multicopy plasmid. As a control, yeast cells were transformed with vector sequences. Since the *TFC2* gene is borne on a plasmid carrying the wild-type *URA3* gene, survival in the absence of this plasmid could be tested by growth on a medium containing 5-fluoroorotic acid (5-FOA), which allows only the cells devoid of the wild-type *URA3* gene to grow (32) (Fig. 3). We found that cells carrying the RPR1–5S hybrid gene on a multicopy plasmid could survive on 5-FOA—i.e., after having lost the wild-type copy of the *TFC2* gene. The absence of the *URA3* plasmid was confirmed by the absence of growth on a medium lacking uracil. Neither the RPR1–5S construct on a centromeric plasmid nor the maxigene construct with the insert in the ICR of the 5S rRNA gene could complement the *tfc2* deletion (data not shown). Therefore, yeast cells can survive with 5S rRNA originating from the RPR1–5S hybrid gene as the sole source of 5S rRNA, provided this gene is present in high copy number. Since supplying yeast cells devoid of TFIIIA with a source of 5S rRNA restored cell growth, we conclude that the only function of TFIIIA essential to cell viability is its participation in 5S rRNA synthesis.

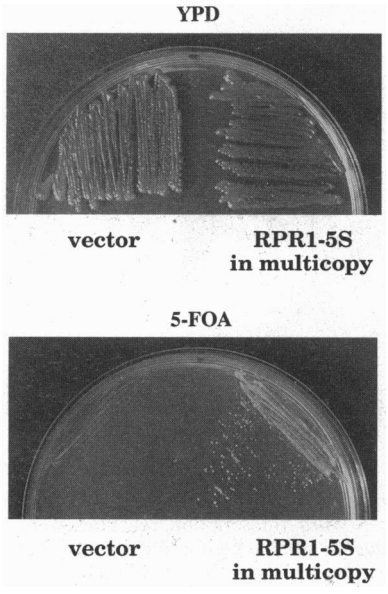

FIG. 3. RPR1–5S construct complements a *tfc2* deletion at 30°C. Yeast strain YRW1, containing a chromosomal deletion of the *TFC2* gene and surviving with a wild-type copy of the *TFC2* gene on a centromeric plasmid, was transformed with the multicopy plasmid pRS-RPR1-5S or with vector sequences alone. Both transformed strains were grown on rich yeast extract/peptone/dextrose (YPD) medium. The ability of these strains to grow in the absence of the centromeric plasmid pJA230 (containing both the *TFC2* and *URA3* genes) was analyzed by streaking the cells on medium containing 5-FOA, which prevents the growth of cells having the wild-type *URA3* gene.

Characteristics of the Cells Devoid of TFIIIA and Surviving with RPR1–5S RNA. Since we had shown previously that in a wild-type strain the majority of 5S RNA originating from the RPR1–5S construct were in the form of precursors (Fig. 2*B*), we asked whether this was still the case in cells having only RPR1–5S RNA as the sole source of 5S RNA. Fig. 4 shows the analysis of small stable RNA in wild-type cells (lanes 1 and 2) and in strain YSC14, devoid of TFIIIA and surviving with the RPR1–5S hybrid gene on a multicopy plasmid (lanes 3 and 4). In wild-type cells, 5S rRNA migrated as a single band of 121 nt. In cells devoid of TFIIIA, the normal 5S rRNA was absent, but two RNA bands 2 and 7 nt longer were observed instead. The total amount of RNA in these two bands detected by ethidium bromide staining was roughly equivalent to the amount of 5S rRNA in wild-type cells. The 5′ ends of these two RNA species, analyzed by primer extension, were identical, homogeneous, and corresponded to a 4-nt extension of the 5′ end of the wild-type 5S rRNA (data not shown). No larger RNA band that could correspond to precursor RPR1–5S RNA was observed (data not shown). All RPR1–5S RNA had therefore been processed in this strain (see comments in *Discussion*).

We found that YSC14 cells grew three times slower at 30°C than did wild-type YRW1 cells (Fig. 5*A*), and cell growth was thermosensitive (Fig. 5*B*). YSC14 cells stopped dividing at 37°C after two or three generations. Retransformation of YSC14 cells with a centromeric plasmid carrying the *TFC2* gene restored a wild-type phenotype (Fig. 5*B*). Microscopic examination of YSC14 cells grown at 30°C showed that many cells had a very elongated form and daughter cells had difficulty separating from mother cells. This phenotype was observed in haploid cells but not in diploids.

DISCUSSION

We have shown in this report that yeast cells can survive with a deletion of the gene encoding TFIIIA provided they have a

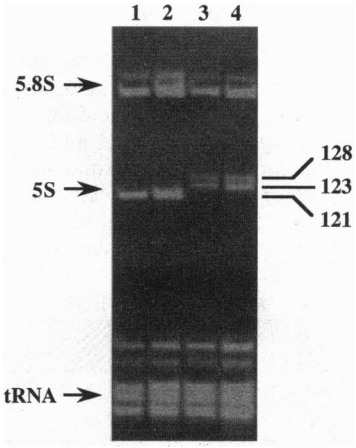

FIG. 4. Analysis of small RNAs in cells surviving with RPR1–5S RNA as the sole source of 5S RNA. Wild-type YRW1 cells and YSC14 cells surviving with the pRS-RPR1-5S plasmid were grown and their content in small RNA was analyzed as described in *Materials and Methods*. Either 5 μg (lanes 1 and 3) or 10 μg (lanes 2 and 4) of total RNA was analyzed for each strain (YRW1, lanes 1 and 2; and YSC14, lanes 3 and 4). The migration of the 5.8S RNA, wild-type 5S RNA and tRNA is indicated. The size of the extended forms of 5S RNA observed in YSC14 were derived from the migration rate of wild-type 5S RNA and the two forms of 5.8S RNA.

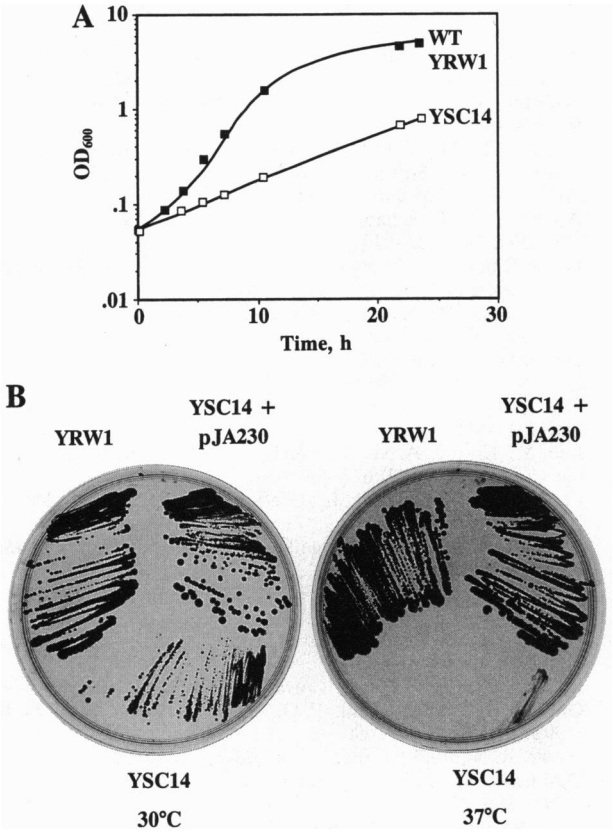

FIG. 5. Growth of cells devoid of TFIIIA. (*A*) The growth in liquid cultures of YSC14 (□) devoid of TFIIIA and surviving with pRS-RPR1-5S was compared with the growth of wild-type (WT) YRW1 (■) at 30°C in rich medium. (*B*) Growth at 30°C and 37°C on rich medium was compared for the wild-type YRW1 strain, the YSC14 strain, and the YSC14 strain retransformed with plasmid pJA230, which carries the wild-type *TFC2* gene.

source of 5S rRNA that does not rely on the presence of active TFIIIA. Therefore, we conclude that the only essential role of TFIIIA in yeast is its participation in the transcription of 5S rDNA. Yeast TFIIIA is not required for the transcription of any other essential gene or for another essential cellular process.

To transcribe the 5S rRNA gene without TFIIIA, we took advantage of the unique organization of the *RPR1* gene. This gene is transcribed as a precursor RNA that is processed at its 5′ and 3′ ends to give rise to the mature *RPR1* RNA (31). Part of the processing reaction removes an 84-nt 5′ leader region which corresponds to the transcribed promoter. In our hybrid construct, the sequence of the mature *RPR1* RNA was replaced by the sequence of 5S rRNA. One advantage of this system is that, for the hybrid gene being transcribed by Pol III, the normal 5S rRNA gene termination signal is used. However, the precursor may not be processed correctly, then impairing the synthesis of wild-type 5S rRNA. No information is yet available on the sequences required for the correct processing of the precursor *RPR1* RNA and *a fortiori* of the RPR1–5S RNA. *In vitro* experiments did not detect any processing of the RPR1–5S hybrid RNA precursor, which was not encouraging. *In vivo*, however, in the context of a cell devoid of TFIIIA (YSC14 strain), a maturation of the RPR1–5S precursor did occur, leading to two RNA species slightly longer than the wild-type 5S rRNA. Primer extension experiments showed that a 4-nt extension was present at the 5′ end of these 5S rRNAs. Therefore, processing of the 5′ leader sequence of *RPR1* occurred slightly upstream of the expected site. Considering the size of the two 5S RNAs present in YSC14, one RNA probably had an additional 3-nt extension at the 3′ end compared with wild-type and the other a 2-nt reduction. Abnormal 3′-end processing is not unexpected since processing of the 3′ end of 5S rRNA requires the pairing of the 5′ and 3′ ends of the RNA (33, 34). The fact that these extended forms of 5S rRNA originating from the RPR1–5S construct were not observed in wild-type cells which contained TFIIIA could be interpreted in different ways. The presence of TFIIIA, which can probably bind to the RPR1–5S RNA, may favor its processing at the expected site or totally prevent processing. Alternatively, in the absence of selective pressure to maintain these abnormal molecules, they may have been directed to a degradation pathway.

Yeast cells devoid of TFIIIA and surviving with the RPR1–5S hybrid gene did not grow as well as wild-type cells. Their growth rate at 30°C was three times slower than that of wild-type cells, and they stopped dividing after two or three cell generations at 37°C. In addition, the cells presented a very elongated shape and did not separate well. This cell morphology is reminiscent of pseudohyphal growth induced under starvation conditions (35). The origin of these defects could be multiple. Even if the only essential function of yeast TFIIIA is its role in the transcription of 5S rRNA genes, TFIIIA could still be involved in other important processes. Yeast TFIIIA binds 5S rRNA like its amphibian homolog and could have a role in the nucleocytoplasmic transport of the RNA. A defect in transport could alter ribosome biogenesis and affect the cell growth rate.

It is probable that the origin of the growth defect in cells lacking TFIIIA arises from the RPR1–5S construct itself. The amount of 5S RNA relative to tRNA and rRNA is not dramatically reduced in cells lacking TFIIIA. However, this reflects an equilibrium state; a limiting amount of 5S RNA probably results indirectly in regulated lower levels of tRNA and large rRNA as well. We have checked that the stability of the slightly extended 5S RNA in YSC14 is not reduced compared with the stability of wild-type 5S RNA (data not shown). Still the rate of synthesis of the precursor and/or its processing rate could be limiting steps. In addition, the RNA produced may be less active than a wild-type 5S RNA due to its modified 5′ and 3′ ends. Part of the primary L1 ribosomal protein binding site resides in the 3′-terminal sequence of 5S RNA paired to its 5′ end (36). The extended 5S RNAs could therefore be defective in L1 binding. Variant 5S RNAs with a slightly altered 3′ end have indeed been shown to bind less well to L1 (34). Since L1 is involved in the assembly of 60S ribosomal subunits (37), a weak binding to L1 could alter ribosomes assembly in YSC14 cells. The defect in ribosome assembly could be even more dramatic at 37°C and result in the observed thermosensitive phenotype.

The system we have developed could be used to study the *in vivo* mechanisms of 5S rRNA synthesis by isolating yeast mutants specifically altered in the production of 5S RNA. The strategy would be similar to the one followed by Nomura and collaborators (38, 39) for the isolation of yeast mutants altered in 35S rRNA production and surviving thanks to rRNA synthesis by RNA polymerase II. This system could also help in understanding 5S rRNA function by facilitating the *in vivo* production and study of mutant 5S RNA species. Nazar and coworkers (40) have already shown that large amounts of mutant 5S rRNA originating from episomal copies of the gene can be produced in yeast. The system described here presents the additional advantage of being able to totally shut off the endogenous production of wild-type 5S rRNA.

We thank J. Segall and her coworkers for the gifts of the *tfc2*-disrupted yeast strain and the plasmids allowing the expression of TFIIIA in yeast and *in vitro*, D. Engelke for providing us with the Pol III expression vectors carrying the 5′ and 3′ sequences of the *RPR1* gene, J. Huet and C. Conesa for the gift of purified Pol III and

transcription factors TFIIIB and TFIIIC, and C. Jackson and P. Thuriaux for their critical reading of the manuscript. S. Camier is a researcher from the Centre National de la Recherche Scientifique. This work was supported by Biotechnology Program Grant B102-CT92-0090 from the European Community.

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