Gene RPA43 in Saccharomyces cerevisiae Encodes an Essential Subunit of RNA Polymerase I*

Pierre Thuriaux†, Sylvie Mariotte, Jean-Marie Buhler, and André Sentenac

From the Service de Biochimie et Génétique Moléculaire, CEA Saclay, Bat. 142, F91191 Gif-sur-Yvette cedex, France

Loan Vu, Bum-Soo Lee, and Masayasu Nomura

From the Department of Biological Chemistry, University of California, Irvine, California 92717

Yeast RNA polymerase I contains 14 distinct polypeptides, including A43, a component of about 43 kDa. The corresponding gene, RPA43, encodes a 326-amino acid polypeptide matching the peptidic sequence of two tryptic fragments isolated from A43. Gene inactivation leads to a lethal phenotype that is rescued by a plasmid containing the 35S ribosomal RNA gene fused to the GAL7 promoter which allows the synthesis of 35S rRNA by RNA polymerase II in the presence of galactose. A screening for mutants rescued by the presence of GAL7-35SrDNA identified a nonsense rpa43 allele truncating the protein at amino acid position 217. [3H]Uridine pulse labeling showed that this mutation abolishes 35S rRNA synthesis without significant effects on the synthesis of 5.8 S RNA and TRNAs. These properties establish that A43 is an essential component of RNA polymerase I. This highly hydrophilic phosphoprotein has a strongly acidic carboxyl-terminal domain, and shows no homology to entries in current sequenced databases, including all the genetically identified components of the other two yeast RNA polymerases. RPA43 mapped next to RPA190, encoding the largest subunit of polymerase I. These genes are divergently transcribed and may thus share upstream regulatory elements ensuring their co-regulation.

The nucleotide sequences(s) reported in this paper have been submitted to the GenBankTM/EMBL Data Bank with accession number(s) U29949.

† To whom correspondence should be addressed: Bât. 142, CEA Saclay, F91191 Gif sur Yvette, cedex, France. E-mail: thuriaux@gnas.saclay.cea.fr.

The abbreviations used are: pol I, II, and III, polymerase I, II, and III; kb, kilobase pair(s).

* The work carried out in M. Nomura’s laboratory was supported by National Institutes of Health Grant R37GM35949. Work done in A. Sentenac’s laboratory was partly funded by Grant BIO2-CT92–0090 from the European Union. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†Towhomcorrespondence shouldbaddressed:Bât.142,CEASaclay,F91191GifsurYvette,cedex,France.E-mail:thuriaux@onas.saclay.cea.fr.

© 1995 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.

It has been established that pre-rRNA synthesis is the only essential function of yeast pol I, since the growth defect of pol I-defective mutants can be bypassed by placing the gene encoding the 35S rRNA under the control of a RNA polymerase II (pol II) promoter (Nogi et al., 1991a). This specialization of pol I enzyme is presumably a general property of eukaryotes.

Biochemical studies have shown that purified preparations of yeast pol I contain 14 distinct polypeptides (Buhler et al., 1976; Valenzuela et al., 1976; Carles et al., 1991). Five of them (ABC27, ABC23, ABC14.5, ABC10α, and ABC10β) are common to all three nuclear RNA polymerases and the corresponding genes (RPB5, RPB6, RPB8, RPB10, and RPC10) have been characterized (Woychik et al., 1990; Woychik and Young, 1990; Treich et al., 1992; Lalo et al., 1993). Two other subunits (AC40 and AC19) are shared by pol I and pol III and are encoded by RPC40 and RPC19, respectively (Mann et al., 1987; Dequard-Chablat et al., 1991). Of the remaining seven pol I subunits, the two large ones, A190 and A135 (encoded by RPA190 and RPA135), have a sequence homology to the two large subunits of pol II, pol III, and bacterial RNA polymerases (Mémet et al., 1988; Yano and Nomura, 1991). These conserved or common nine subunits are essential for growth, as shown by the mutational inactivation of their genes (Young et al., 1991 and Thuriaux and Sentenac, 1992, and references therein). Remarkably, all but two of them (ABC14 and ABC10α) also have a structural equivalent in archael RNA polymerases (Langer et al., 1995).

This conservation obviously suggests that the corresponding polypeptides fulfill functions that are common to the central mechanism of transcription itself. Another subunit A12 (encoded by RPA12 or RN4) has some homology to the B12.6 subunit of pol II (encoded by RPB9). RPA12 and RPB9 have been shown to be only conditionally essential (Woychik et al., 1991; Nogi et al., 1993).

In addition to this conserved or even common core of subunits shared by all three RNA polymerases, four polypeptides, A49, A43, A34, and A14, are found in pol I preparations only. The role of these polypeptides in the synthesis of rRNA is still poorly understood, but there is evidence that they are not strictly required for the catalytic activity of yeast pol I. Indeed, disrupting the A34- or A14-encoding genes hardly affects cellular growth (Smid et al., 1995), whereas a pol I preparation lacking both A49 and A43 retained catalytic activity in vitro as tested on nonspecific DNA templates (Hager et al., 1977). A49 has since then been shown to be a genuine pol I subunit that is partly dispensable in vivo (Liljelund et al., 1992). In the case of A43, the possibility of a fortuitous co-purification of that polypeptide with pol I could not be ruled out so far. The present work establishes that A43 is indeed an essential and specific
Yeast RNA Polymerase I Subunit

Plasmids and Strains—The RPA43 inserts isolated in the present work originated from three distinct laboratory strains of *S. cerevisiae*, X2180, AB320, and DBY476. The *A43-1* clone was previously isolated by screening of a lambda 11 genomic library prepared from strain X2180 using anti-A43 antibodies (Riva et al., 1988). A 2.4 kb HindIII-EcoRI fragment of *A43-1* was subcloned in *pBluescriptKS* (+) (Stratagene) to generate pA43, whereas YCPA43-3 and YCPA43-6 were constructed by subcloning a BamHI fragment of 1.9 kb in YCP50, pA43-L-LEU was generated by creating an additional BglII site a few base pairs downstream of the putative initiator triplet of *RPA190* derived from strain D101, and a 3.5 kb fragment including the proximal two-thirds of the RPA43 coding region was removed. The linearized plasmid containing the remaining portion was transferred into the mutant strains, generating Leu*+/Ura+* transformants which were selected on galactose medium and screened for non-growth phenotype on glucose medium. Plasmids were prepared from transformants that were unable to grow on glucose medium, and pNOY272 derivatives that failed to complement the rpa43-1 mutation were recovered in E. coli. The rpa43-1 mutation was identified by sequencing both strands of the entire open reading frame of the RPA43 gene in these plasmids.

Analysis of RNA Labelled in Vivo—Cells were grown in synthetic galactose medium supplemented with casamino acids, tryptophan, and adenosine at 30°C to a cell density (A600) of approximately 0.2. Each culture was divided into two parts. Glucose was added to one culture to a final concentration of 2%, and the other culture received water, serving as control. After incubation at 30°C for 1 h, [3H]uridine (174 mCi/mmol; 100 mCi/ml) was added, and after a 30-min incubation the labeling was stopped by immersing culture flasks into a dry ice-ethanol bath. RNA was isolated and analyzed by electrophoresis on a 2% polyacrylamide, 0.5% agarose composite gel followed by autoradiography as described previously (Nogi et al., 1991a).

RESULTS

Sequence Determination and Physical Linkage between RPA43 and RPA190—RPA43 was first isolated as a *A43-1* clone constructed from the *S. cerevisiae* strain X2180. This clone, obtained by immunological screening of the lambda 11 bank with polyclonal antibodies raised against A43, produced a polypeptide of 43 kDa (as determined by SDS-polyacrylamide gel electrophoresis) when expressed in Escherichia coli (Riva et al., 1986). A 2.4 kb EcoRI-HindIII fragment of the *A43-1* insert was subcloned in *pBluescriptKS* (+), yielding plasmid pA43 (Fig. 1). The DNA sequence of this fragment and of the corresponding regions derived from two other yeast strains, AB320 and DBY476 (see legend of Fig. 1), was determined (see below for the sequence obtained from pNOY272 and pNOY273). The contiguous 2097-base pair DNA sequence (accession number U22949) generated from these determinations contained a 978-base pair uninterrupted open reading frame encoding a 326-amino acid polypeptide. The predicted amino acid sequence is identical in all three strains, but AB320 differs from the other two by a silent G → A transition at nucleotide 546 of the open reading frame. There is a perfect match with the amino acid sequences of two peptides (SQAESLPVSNK and ILDAPDL, underlined in Fig. 2) which were isolated from the purified A43 protein after tryptic digestion and whose amino acid sequences were directly determined. This confirms the original identification of the gene (hereafter called RPA43) based on immunological methods using specific antibodies against A43. The absence of the (A/T)ACTAAC consensus motif present at the branch point of all *S. cerevisiae* introns identified so far strongly argues that RPA43 is an intron-less gene.

In the course of the sequence analysis of RPA43, we found that its initiator ATG is separated by 805 base pairs from a divergently transcribed reading frame, that turned out to be RPA190 (Mémet et al., 1988), encoding the largest subunit (A190) of pol I (Fig. 1). As previously noted, the RPA43-RPA190 intergenic region contained putative RPG and PAC boxes (Mémet et al., 1988; Dequard-Chablat et al., 1991) that could be involved in regulation of the transcription of these genes. This physical linkage cannot be due to an artifact generated by some in vitro recombination event during the preparation of the

3 M. Riva, personal communication.
Yeast RNA Polymerase I Subunit

RPA43 Is an Essential Gene Required for rRNA Synthesis—

The rpa43Δ::LEU2 deletion, removing most of the RPA43 coding sequence (Fig. 1), is recessive lethal. This conclusion was first obtained by tetrad analysis using a diploid strain (D101) carrying rpa43Δ::LEU2 on one chromosome and RPA43 on another chromosome. Tetrads showing the 2:2 segregation of viable (RPA43) and non-viable (rpa43Δ::LEU2) spores were observed (Fig. 3, left panel). As such, this lethal phenotype could be limited to a defective spore germination phenotype, or due to a negative effect of the deletion on the expression of adjacent RPA190. Both interpretations were ruled out by showing that viable rpa43Δ::LEU2 segregants can be recovered in the D101 spore isolate when the latter strain bears the centromeric plasmid YCPA43-12 (URA3 RPA43) (Fig. 2, middle panel).
such mutants, NOY639 and NOY640. To clone and characterize the complementation group were recovered in most asci, indicating the complementation of text for further explanations). Right panel, tetrads derived from D101 bearing the YCPA43-12 (CEN4 URA3RPA43) plasmid. More than two viable spores were recovered in most asci, indicating the complementation of rpa43::LEU2 by the YCPA43-12 (CEN4 URA3RPA43) plasmid (see text for further explanations). Right panel, tetrads derived from D101 bearing pNOY102 (URA3, GAL7-35SrDNA). Spores were germinated on YPGal. Note the mendelian segregation of two fast-growing (RPA43) and two slow-growing segregants, the latter bearing the rpa43::LEU2 with partial rescue of growth defect due to the (inefficient) synthesis of 35S rRNA from the GAL7-35SrDNA fusion gene (Nogi et al., 1991a).

panel). As expected, the segregants carrying rpa43::LEU2 invariably harbored the URA3 RPA43 plasmid, and could not lose it as evidenced by their inability to form colonies on 5-fluoroorotic acid medium which selects for the loss of the URA3 plasmid.

The complementation data described above show that A43 is essential for cell growth, but do not prove that this protein is required for rRNA synthesis and thus is a functional component of pol I. The proof was obtained by the demonstration that the lethal phenotype of the RPA43 deletion can be rescued by the presence of pNOY102, which carries the GAL7-35SrDNA fusion gene (Nogi et al., 1991b). This fusion gene allows the synthesis of the 35S rRNA by pol II from the GAL7 promoter in the presence of inducer galactose. As shown in Fig. 3 (right panel), spores isolated from strain D101(pNOY102) and germinated on YPGal have a 2:2 segregation of two fast-growing RPA43 spores, and two slow-growing spores corresponding to the rpa43::LEU2 segregants rescued by the GAL7-35SrDNA fusion. The latter segregants failed to grow when replica plated on glucose containing YPD medium and were also unable to grow on galactose medium in the presence of 5-fluoroorotic acid, confirming that their growth depends on the presence of this plasmid.

Isolation of the rpa43-1 Mutation—Mutants that depend on the presence of the GAL7-35SrDNA gene for growth were isolated and classified into complementation groups by genetic crosses (Nogi et al., 1991b) and the genes representing some of these complementation groups were cloned and characterized (Nogi et al., 1993; Nomura et al., 1993; Keys et al., 1994). Complementation group RNR12 was previously defined by two such mutants, NOY639 and NOY640.4 To clone and characterize the corresponding gene, a yeast genomic library (prepared from strain DBY746) was introduced into mutant NOY639, and transformants able to grow on glucose media (where the GAL7 promoter is repressed) were recovered. A plasmid recovered from one of them (pNOY286) was analyzed further. Sequencing of the subcloned DNA fragments (pNOY272 and pNOY273; see Fig. 1) responsible for the complementation revealed the presence of the amino-terminal portion of the RPA190 gene (in pNOY272) and an open reading frame containing the first 308 codons of the RPA43 coding sequence, but ending with GATC (nucleotide 921–924; the Ala of the first ATG of the RPA43 gene) was amplified by the polymerase chain reaction (PCR) and sequenced. The latter open reading frame is the +1 followed by the sequence of the original cloning vector adjacent to the BamHI site (GGATCC) for construction of the DNA library. Thus, the RPA43

4 L. Vu, K. Sutton, and M. Nomura, unpublished experiments.

insert carried by the original complementing plasmid (pNOY286) as well as its derivatives are actually bearing a mutant form of RPA43, that encodes A43 down to the isoleucine residue at position 308 but lacks the carboxy-terminal 18 amino acids. In pNOY273, the missing 18 amino acids are replaced by four amino acids, PGIH, translated from nucleotide sequence derived from the multicloning site of the vector (pRS314) used to construct this plasmid. This generated a small decrease (about 1.5 kDa) in the size of A43 which was confirmed by Western immunoblot analysis of extracted protein prepared from rn12 mutants carrying pNOY273 (data not shown). The growth rates of the rrn12 mutants carrying pNOY273 was only slightly (about 10%) slower than the growth rate of the parent wild type strain, and the carboxy-terminal 18-amino acid portion is therefore not critical to the function of A43.

The mutation carried by mutant NOY639 was recovered by gap repair using plasmid pNOY272 carrying the cloned RPA43 (missing the COOH-terminal 18 amino acids), as described under “Materials and Methods.” The mutation carried by both NOY639 and NOY640 was identified to be a G to A transition at nucleotide +650 that changes the UGG Trp codon at amino acid position 217 to a UAG nonsense codon. The results demonstrate that the rrn12 mutation is in fact in the RPA43 gene, and that truncation of A43 (362 amino acids) at the position distal to His-216 by a nonsense mutation leads to lethality to yeast cells. The presence of the truncated A43 protein fragment in the mutant cells (under permissive growth conditions, that is, mutant cells growing in galactose medium using the GAL7-35SrDNA fusion gene and pol II) was, in fact, demonstrated by Western immunoblot analysis of extracts using antiserum against A43, and its size relative to the size of A43 was roughly consistent with the position of the nonsense codon (data not shown). We have not examined the question of whether (defective) pol I missing the intact A43 exists stably in these mutant cells, and if so, the observed truncated A43 fragment is associated with the defective pol I. In any event, we renamed RRN12 as RPA43 and the mutation carried by NOY639 and NOY640 is now designated as rpa43-1.

Direct Demonstration of Defects in 35S rRNA Synthesis in rpa43-1 Mutant Strain—As described above, mutants carrying rpa43::LEU2 or rpa43-1 fail to grow on glucose media, but were able to grow on galactose media, if they carried the GAL7-35SrDNA fusion gene on a suitable multicopy plasmid. This indicated that A43 is essential for 35S rRNA synthesis by pol I in vivo. We confirmed this conclusion directly by [3H]uridine pulse-labeling experiments using a rpa43-1 mutant strain. Both the mutant (NOY639) and the control wild-type (NOY418) strains were grown in synthetic galactose medium, and [3H]uridine pulse labeling experiments were carried out with and without prior repression of the GAL7 promoter by glucose, as was done in previous studies (Nogi et al., 1991a, 1991b). As shown in Fig. 4, synthesis of large rRNA (18 S, 25 S, 5.8 S, and other precursor rRNAs) was strongly inhibited by glucose relative to synthesis of 5 S RNA and tRNAs, showing that rpa43-1 abolishes the synthesis of large rRNA by pol I specifically without significant effect on 5 S RNA and tRNA synthesis.

DISCUSSION

It has been known that highly purified preparations of yeast pol I, but not pol II or pol III, contained the A43 protein. However, some pol I preparations lacking both A49 and A43 retained catalytic activity as assayed with nonspecific DNA template (Hager et al., 1977). Although A49 has since then been shown to be a genuine subunit of pol I (Liljelund et al., 1992), the possibility of a fortuitous co-purification of A43 with pol I could not be ruled out. We have now sequenced and
rRNA synthesis in the mutant was inhibited by glucose, as was that of autoradiograms obtained after longer gel exposures. This indicates that 5.8S composite gel. An autoradiogram of the dried gel is shown. We note that 24256 using a samples containing equal amounts (approximately 1 transcription by pol II in the presence of inducer galactose. The medium. At a cell density (A600 of approximately 0.2, cultures were divided into two parts: and glucose (final concentration 2%) was added to one part (lanes 2 and 4), the other received water, serving as control (lanes 1 and 3). At 1 h after glucose addition, cells were pulse-labeled with [3H]uridine for 30 min. RNA was isolated from each culture, and samples containing equal amounts (approximately 1 x 10^6 cpm) of [3H]RNA were analyzed by electrophoresis on a polyacrylamide-agarose composite gel. An autoradiogram of the dried gel is shown. We note that autoradiograms obtained after longer gel exposures indicated that 5.8S rRNA synthesis in the mutant was inhibited by glucose, as was that of 18S and 25S rRNAs.

characterized the corresponding gene, RPA43. Its inactivation by a nonsense mutation or by an extended internal deletion is lethal, but cells carrying these mutations can be rescued by introduction of the GAL7-35S-rDNA fusion gene and its transcription by pol II in the presence of inducer galactose. The results indicate that the absence of intact A43 leads to failure to synthesize 35S rRNA using pol I, and this conclusion was confirmed directly by [3H]Juridine pulse labeling experiments using a rpa43 nonsense mutant. Thus, A43 is essential for 35S rRNA synthesis by pol I in vivo, and hence, must represent an essential subunit of pol I, even if it does not appear to be required for pol I-dependent transcription of nonspecific DNA templates (Hager et al., 1977). It will be interesting to re-investigate the pol I preparations lacking A43 using in vitro transcription assays that allow specific transcription initiation of the natural pol I promoter (Lue and Kornberg, 1990; Riggs and Nomura, 1990; Schultz et al., 1991; Keys et al., 1994).

Genes encoding distinct subunits of the same heteromultimeric enzyme are usually dispersed on the yeast genome, and this also applies to RNA polymerase subunits (Young, 1991; Thuriaux and Sentenac, 1992). Surprisingly, however, RPA43 is physically linked to RPA190. The two genes are divergently transcribed, and their coding regions are separated by 805 base pairs containing putative RPG and PAC boxes (Mémet et al. 1988; Dequard-Chablat et al., 1991). Thus, as in the case of the L46-L24 ribosomal protein gene pair (Kraakman et al., 1989), these two genes might be co-regulated using common upstream activating sequences such as the putative RPG and PAC boxes, thereby helping balance synthesis of these two essential pol I subunit proteins.

Early studies showed that A43 is not immunologically related to yeast pol II or pol III or their purified subunits, suggesting that A43 is unique to pol I (Huet et al., 1982). The present study extends this conclusion by showing that A43 has no significant sequence homology to any other entries in current data banks and is, in particular, unrelated to any of the 12 subunits of yeast pol II and to the 14 genetically characterized subunits of yeast pol III (Young, 1991), Thuriaux and Sentenac (1992), and Sadhale and Woychik (1994), and references therein. However, there is a poorly characterized polypeptide of 37 kDa, C37, that is present in some catalytically active preparations of yeast pol III and might thus be a genuine subunit of that enzyme (Huet et al., 1985). The corresponding gene has not yet been identified.

As mentioned in the Introduction, there are three other subunits, A49, A34, and A14, in addition to A43, which are unique to pol I. However, the genes for A49, A34, and A14 can be disrupted with only limited adverse effects on cell growth (Liljelund et al., 1992; Nogi et al., 1993; Smid et al., 1995), while the gene for A43 is essential. Therefore, A43 appears to be special and may have some important function(s) specific to pol I. For example, it may directly interact with the yeast equivalents of the SL1 and UBF1 initiation factors that are well characterized in vertebrate in vitro transcription systems (Reeder, 1992; Eberhard et al., 1993; Radebaugh et al., 1994; Zomerdijk et al., 1994), or to the products of several RNR genes (Keys et al., 1994) that are known to be specifically required for pol I activity in vivo or some other unidentified pol I-specific regulatory factors. A similar role was proposed for a complex of three pol III-specific subunits, C82, C34, and C31 (Thuriaux and Sentenac, 1992; Werner et al., 1993). Curiously, A43 and the C31 subunit of yeast pol III (Mosrin et al., 1990), although otherwise unrelated, share a strongly acidic COOH-terminal domain of about 40 residues. Deleting the last 16 amino acids of C31 leads to a conditional growth phenotype and produces a mutant enzyme that is affected in transcription initiation, but not termination or elongation, suggesting that it could be altered in its interaction with components of the pol III-specific initiation factor TFIIIB (Thullier et al., 1995). In the case of A43, the functional significance of the acidic tail is unclear.

Removing the COOH-terminal 18 amino acids representing nearly half of the acidic tail gives only a weak negative effect on its function as judged by the ability of pNOY273 (and other related plasmids) to complement the rp43-1 mutation. Another interesting feature of A43 is that it is a phosphoprotein with an average of four phosphorylated residues per molecule (Bréant et al., 1983). With the complete amino acid sequence of A43 now deduced from the nucleotide sequence, it becomes possible to identify the phosphorylated residues and then design experiments to examine the functional and/or regulatory significance of the phosphorylation, thereby clarifying the function of this essential component of the rRNA synthesis machinery.

Acknowledgments—We thank Karen Sutton for her participation in isolation and complementation analysis of two rnr12(rp43) mutants described in this paper, Claire Boschiero, Catherine Daia, and Eric Quémeneur for their contribution to RNA sequencing, and Michel Riva for communicating the peptidic sequence of the two tryptic fragments of A43.

REFERENCES

Boeke, J. D., Lacroute, F. & Fink, G. R. (1984) Mol. Gen. Genet. 197, 345–346

Brehant, B., Buhler, J. M., Sentenac, A. & Fromageot, P. (1983) Eur. J. Biochem. 130, 247–251

Buhler, J. M., Iborra, F., Sentenac, A. & Fromageot, P. (1976) J. Biol. Chem. 251, 1712–1717

Carles, C., Treich, I., Bouet, F., Riva, M. & Sentenac, A. (1991) J. Biol. Chem. 266, 24092–24096

Dequard-Chablat, M., Riva, M., Carles, C. & Sentenac, A. (1991) J. Biol. Chem. 266, 15300–15307

Eberhard, D., Tora, L., Egly, J. M. & Grummt, I. (1993) Nucleic Acids Res. 21, 24092–24096

FIG. 4. Polyacrylamide-agarose gel electrophoresis of RNA synthesized in a rpa43-1 mutant (NOY639) and the parent (NOY418) strains growing in galactose with and without prior glucose addition. Parent strain NOY418 (lanes 1 and 2) and rpa43-1 mutant NOY639 (lanes 3 and 4) were grown at 30°C in galactose medium. At a cell density (A600) of approximately 0.2, cultures were divided into two parts: and glucose (final concentration 2%) was added to one part (lanes 2 and 4), the other received water, serving as control (lanes 1 and 3). At 1 h after glucose addition, cells were pulse-labeled with [3H]uridine for 30 min. RNA was isolated from each culture, and samples containing equal amounts (approximately 1 x 10^6 cpm) of [3H]RNA were analyzed by electrophoresis on a polyacrylamide-agarose composite gel. An autoradiogram of the dried gel is shown. We note that autoradiograms obtained after longer gel exposures indicated that 5.8S rRNA synthesis in the mutant was inhibited by glucose, as was that of 18S and 25S rRNAs.
