Yeast *RPO41* gene product is required for transcription and maintenance of the mitochondrial genome

(RNA polymerase/gene disruption/nuclear gene/DNA replication)

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**ABSTRACT** A 4-kilobase DNA fragment carried by a recombinant *Agt11* bacteriophage appears to contain most of the coding information for the 145-kDa subunit of the *Saccharomyces cerevisiae* mitochondrial RNA polymerase. The *RPO41* gene is located on chromosome VI, as determined by hybridization to electrophoretically separated yeast chromosomes. Hybridization and gene disruption/replacement experiments show that the *RPO41* gene exists in a single copy and that its product is required for transcription and maintenance of the mitochondrial genome.

We have purified a 145-kDa oligoribonucleotide synthetase that appears to be a subunit of the yeast mitochondrial RNA polymerase (J. L. K. and I. R. L., unpublished data). To verify that the enzyme is indeed mitochondrial, we sought to identify mutations that inactivate the enzyme and then show that the mitochondria are specifically affected as a consequence of such mutations. We, therefore, cloned the gene encoding the 145-kDa RNA polymerase subunit (unpublished data). The gene was retrieved as a 4-kilobase (kb) genomic yeast DNA fragment carried by a recombinant *Agt11* bacteriophage, phage 102 (Fig. 1). The yeast DNA insert in phage 102 encoded an approximately 140-kDa polypeptide that was not, however, a fusion protein with β-galactosidase. Consequently, the 4-kb yeast DNA insert represents all or nearly all of the protein coding region of the gene for the RNA polymerase subunit.

In this paper we describe the further characterization of the gene for the 145-kDa polypeptide. We also describe biochemical and genetic experiments that show that in addition to transcription, the nuclear-encoded enzyme is also required for maintenance of the mitochondrial genome.

**MATERIALS AND METHODS**

**Microbial Strains and DNA.** Standard analyses of phage and plasmid DNAs were performed according to Maniatis *et al.* (2). Gel blot hybridization analysis (3) was performed using GeneScreenPlus membranes (New England Nuclear) according to the manufacturer's directions with minor alterations.

Yeast strains were grown on rich (YPD) or appropriate minimal media as described (4). Yeast DNA was isolated by a rapid small scale method (5) with minor modifications. A *ura3* diploid strain (α/α, *ura3*-52/*ura3*-52, *lys2*-801/*lys2*-801, ade2-101/ade2-101, +/*his3 Δ500, +/*pyr1, trp1-Δ901/+) kindly given to us by Ronald Davis, Stanford University, was transformed with *URA3* DNA by the lithium acetate procedure (6). Ura" transformants were sporulated, and tetrads were analyzed as described by Sherman *et al.* (4).

**RESULTS**

A Single-Copy Nuclear Gene Product Is Required by Mitochondria. One-step gene disruption experiments were performed (9) to address two questions: (i) Is the gene for the 145-kDa subunit of mitochondrial RNA polymerase (the yeast DNA insert of phage 102) a single-copy, essential gene? (ii) Is the gene product required uniquely by the mitochondria?

The *URA3* inserts in the *RPO41* gene fragment carried by phage 102 were constructed by M. Snyder, Stanford University (7). Briefly, an internally deleted Tn10 transposon derivative (del16del17 of Foster et *al.* (8)) was modified by inserting the yeast *URA3* gene near one end. The resulting Tn/*URA* transposon, which was still tetracycline resistant, was used in vivo for insertional mutagenesis of phage 102 (unpublished data) by selecting for tetracycline resistant lysogens. The resulting insertion-carrying mutants of phage 102 were screened by restriction mapping to identify those with the approximately 4-kb Tn/*URA* inserts in the yeast *RPO41* gene fragment. DNAs from two such Tn/*URA*-carrying phages (phages 6 and 12) were used for the gene disruption experiments.

**FIG. 1.** Restriction map of phage 102 yeast DNA insert and the corresponding genomic region. The open box in phage 102 represents the approximately 4-kb yeast DNA fragment inserted in the *Agt11* EcoRI site near the carboxyl terminus of the *lacZ* gene (ref. 1; J. L. K. and I. R. L., unpublished data). Restriction endonuclease sites are indicated on the thick line representing the yeast genome. EcoRI (R), HindIII (H), and Bgl II (B) sites are shown. Coordinates on the bottom line are in kb, with the right-most EcoRI site designated zero. The lower-case h at ca. -0.9 is a polymorphic HindIII site present in the phage 102 yeast DNA insert but absent from some other alleles of the RNA polymerase gene. Note that the EcoRI sites at both ends of the 4-kb insert correspond to normal genomic EcoRI sites. The triangles above the 102 insert represent *Tn/*URA* insertions 6 and 12; note that the actual size of these inserts is approximately 4 kb.

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Abbreviation: kb, kilobases.

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A DNA fragment containing a selectable marker (URA3) was introduced into the RNA polymerase gene fragment in phage 102 by M. Snyder (personal communication) using a modified Tn10 transposon carrying the yeast URA3 gene (referred to below as Tn/URA inserts) (7). Two useful phages resulting from this procedure that carried Tn/URA inserts in the yeast DNA are indicated in Fig. 1. In extracts of induced lysogens of Escherichia coli, Tn/URA insert 6 dramatically reduced, and Tn/URA insert 12 modestly reduced accumulation of RNA polymerase antigen (data not shown but see ref. 7). Thus, both insertions reduced expression of the RNA polymerase gene in E. coli, presumably by disrupting protein coding sequences. Since the Tn/URA insertions 6 and 12 were between the two Bgl II sites at coordinates −0.3 and −3.3 (Fig. 1) and since the Tn/URA insert lacks Bgl II sites, Bgl II digestion of either phage liberates a linear fragment (approximately 7 kb) suitable for gene disruption.

A diploid ura3 strain was transformed with DNA carrying either Tn/URA insertion 6 or 12 that had been cleaved with Bgl II, and Ura+ transformants were selected. Several diploid transformants from each experiment were sporulated, tetrads dissected, and the individual spores grown on standard rich medium (YPD). Under these conditions, the expected tetrad for a single-copy essential gene whose product is not required uniquely in the mitochondria will contain two viable spores (Ura−) and two nonviable spores (Ura+, disrupted in the RNA polymerase gene by the Tn/URA insertion). However, tetrads with four viable spores were obtained (Table 1).

The properties of these spores were consistent with the idea that the RNA polymerase whose gene had been disrupted is required only in mitochondria. In every case, two spores were Ura− and two Ura+ as expected. On the other hand, the two Ura+ spores always produced smaller colonies (slower growth) on standard medium than the Ura− spores. In addition, the two Ura− colonies were pink or red as expected (since the parental Ura− diploid was homozygous for ade2 that results in accumulation of a red pigment during respiration (10)), whereas the two Ura+ colonies were white. The slow growth and white color (in an ade2 background) of the Ura+ spores are two properties of petite mutants, which lack functional mitochondria and, therefore, are defective in respiration. To test the idea that the Ura+ (gene disrupted) spores were indeed petites, we examined their growth on minimal medium containing glycerol as sole carbon source. Indeed, the Ura+ spores did not grow on glycerol while their Ura− siblings did (Table 1). Identical results were obtained for both Tn/URA insertions 6 and 12 and for tetrads from several independent transformants of each. Therefore, disrupting the RNA polymerase gene results in defective respiration, presumably due to loss of functional mitochondria. Note that with glycerol as sole carbon source, the expected 2:2 segregation of viable:inviable spores was observed, demonstrating that the RNA polymerase gene is a single copy nuclear gene and is essential under these specific growth conditions.

To reinforce this conclusion, it was necessary to show that the Tn/URA constructs had actually inserted into and disrupted the RNA polymerase gene. For one tetrad each in the experiments using Tn/URA insertions 6 and 12, whole genomic DNAs from the diploid transformant and the Ura− and Ura+ segregants were prepared, digested with Bgl II, and analyzed by gel blot hybridization (3). The probe used (the large EcoRI fragment of the phage 102 insert) hybridized, as expected, to two Bgl II fragments from the intact gene in the diploid transformant: the 3-kb Bgl II fragment from coordinates −0.3 to −3.3 and the 5.6-kb Bgl II fragment from coordinates −3.3 to −8.9 (Fig. 2). In addition, the probe detected a new band of 7 kb not seen in the wild-type parental DNA; this 7-kb band was presumably composed of the 3-kb Bgl II fragment plus the 4-kb Tn/URA insert. Consistent with this idea, both of the Ura+ haploid segregants lacked the 3-kb (wild-type) band but contained the 7-kb (insertionally mutagenized) RNA polymerase gene band. The Ura− segregants (only one shown) contained the wild-type 3-kb fragment but lacked the larger, insertionally mutagenized fragment. These results demonstrate that the gene disruptions specifically affected the RNA polymerase gene. Since this is the first gene to be described for a subunit (probably the largest) of a fourth class of nuclear-encoded RNA polymerase, we refer to it as the RPO41 gene (11).

Further Analysis of the RPO41 Gene. We confirmed that the polymerase gene is nuclear and mapped it to a specific chromosome by hybridizing part of it (the yeast insert of phage 102) to a blot of electrophoretically-separated yeast chromosomes (provided by D. Mandoli and D. Vollrath). It was found to hybridize to chromosome VI (Fig. 3). Our earlier findings (J.L.K. and I.R.L., unpublished data) suggested that the 4-kb yeast DNA insert carried by phage...
102 contained most, if not all, of the coding sequences of the RPO41 gene. To determine whether this DNA fragment contained either or both ends of the gene, the following functional test was performed. The two EcoRI fragments of the gene (2.7 kb and 1.3 kb) were subcloned separately into the EcoRI site of the transformation vector YIp5 (URA3) (13). Whereas transformation of a haploid strain (ura3, RPO41) with an internal fragment of RPO41 will result in two disrupted polymerase genes, transformation with a fragment with one end intact will result in one intact and one disrupted RNA polymerase gene (14). If either EcoRI fragments contains an end of the gene, transformation with that plasmid should yield transformants with one intact gene copy; on standard plates these should all give rise to red colonies in an ade2 background (not petites). On the other hand, if either fragment is internal to the gene, all transformant colonies should be white (petites), since no intact gene will be present. The results showed that the left, large (2.7 kb) EcoRI fragment contained an intact end of the RPO41 gene (all red colonies) whereas the right, small (1.3 kb) EcoRI fragment did not (all white colonies); the right end of the gene must, therefore, lie to the right of coordinate zero (Fig. 1) (data not shown). Thus, the Tn/URA inserts described above clearly disrupt the functional RPO41 gene.

Besides synthesizing the various mitochondrial RNA species, mitochondrial RNA polymerase is apparently also involved in priming mitochondrial DNA replication at some origins (15, 16). This fact leads to the prediction that disrupting the RPO41 gene in a haploid strain will abolish mitochondrial DNA replication. To test this idea, we grew the appropriate strains under conditions in which functioning mitochondria are normally present but are not required for survival and then tested for the presence of mitochondrial DNA. Fig. 4 shows that an rpo41 haploid lacks detectable mitochondrial DNA as assayed by dye/cesium chloride gradients. Furthermore, mitochondrial DNA was undetectable in the rpo41 haploids by probing Southern blots of total DNA with a mitochondrial DNA probe (Fig. 5). It has been shown that mitochondrial protein synthesis is required for the propagation of wild-type mitochondrial DNA in yeast (20). Since the product of the RPO41 gene may be required for protein synthesis we cannot conclude, based on our present data, that the gene product is directly required for mitochondrial DNA replication. However, we can conclude that the product of the RPO41 gene is required for maintenance of the mitochondrial genome.

**DISCUSSION**

Yeast mitochondrial RNA polymerase has been purified by several different procedures, yielding preparations with
greatly differing properties (21–24). Recent work suggests that the enzyme may be composed of two or more separable components, one of which can catalyze the synthesis of RNA chains but cannot recognize promoter sequences (analogous to bacterial "core" enzyme) and others that interact with the core component to facilitate promoter recognition (analogous to bacterial σ factors) (25). The 145-kDa subunit encoded by the RP041 gene probably represents part or all of the core enzyme (J.L.K. and I.R.L., unpublished data).

Our experiments have shown that the RP041 gene product is required by the mitochondria and that, in addition to its role as the transcriptase for this organelle, it is required for maintenance of the mitochondrial genome. It will be interesting to see whether the RP041 gene product is required for replication of petite genomes consisting of simple sequence DNA (26).

Gene replacement experiments with RP041 subclones showed that the fragment of the gene retrieved as the insert in phage 102 contains the left end of the gene and most of the coding information for the 145-kDa subunit but lacks the right end of the gene (as drawn in Fig. 1). This information makes it possible to design in vitro mutagenesis/transposition experiments that could generate conditional lethal mutants of the locus (27). Studies of these mutants should help elucidate the specific role that the 145-kDa subunit plays in RNA and DNA synthesis. In addition, selection of extragenic suppressors (pseudorevertants) of the conditional lethal mutants should lead to identification of other genes whose protein products interact with the 145-kDa subunit in vivo (14); one such gene should encode the σ factor analogue of the mitochondrial RNA polymerase.

The molecular weight of the RP041 gene product is strikingly similar to β and β' subunits of eubacterial RNA polymerases and the large subunits of eukaryotic nuclear RNA polymerases (ref. 28 and references therein). This similarity may not be coincidental. Experiments using antibodies against individual subunits of eukaryotic nuclear RNA polymerases have suggested that the largest subunits of RNA polymerases I, II, and III are structurally related (28). Furthermore, experiments utilizing these antibodies suggest that there is antigenic relatedness between the large subunits of the eukaryotic nuclear RNA polymerases and the large subunits of eubacterial (29, 30) and archaeabacterial (30) RNA polymerases. DNA sequence analysis of cloned genes for the largest subunits of eukaryotic RNA polymerase II (31–33) and RNA polymerase III (32) indicating extensive amino acid sequence homology among the largest subunits of the eukaryotic and the eubacterial RNA polymerases confirms this suggestion. The RP041 gene product may be related to these other eu- and prokaryotic polymerase subunits, since our results (A.L.G., unpublished observations) indicate that the affinity purified IgGs that react with the mitochondrial 145-kDa subunit (J.L.K. and I.R.L., unpublished data) also react with one of the large subunits of E. coli RNA polymerase, probably β'. If sequence analysis of the cloned RP041 gene confirms this apparent structural homology, the idea of evolutionary conservation in the structure of the transcription apparatus will be even more firmly established. Finally, the yeast RP041 gene might prove useful as a hybridization probe with which to isolate homologous genes from other sources (for example, the chloroplast RNA polymerase subunit gene) (34).

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