The C-terminal Region of Mitochondrial Single-subunit RNA Polymerases Contains Species-specific Determinants for Maintenance of Intact Mitochondrial Genomes

Thomas Lisowsky,^{*†} Detlef Wilkens,^{*} Torsten Stein,[‡] Boris Hedtke,[§]
Thomas Börner,[§] and Andreas Weihe[§]

^{*}Botanisches Institut, Heinrich-Heine-Universität Düsseldorf, D-40225 Düsseldorf, Germany;

[‡]Department of Pathology, University of Glasgow, Western Infirmary, Glasgow, G11 6NT, United Kingdom; and [§]Institut für Biologie, Humboldt Universität, D-10115 Berlin, Germany

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Functional conservation of mitochondrial RNA polymerases was investigated *in vivo* by heterologous complementation studies in yeast. It turned out that neither the full-length mitochondrial RNA polymerase of *Arabidopsis thaliana*, nor a set of chimeric fusion constructs from plant and yeast RNA polymerases can substitute for the yeast mitochondrial core enzyme Rpo41p when expressed in $\Delta rpo41$ yeast mutants. Mitochondria from mutant cells, expressing the heterologous mitochondrial RNA polymerases, were devoid of any mitochondrial genomes. One important exception was observed when the carboxyl-terminal domain of Rpo41p was exchanged with its plant counterpart. Although this fusion protein could not restore respiratory function, stable maintenance of mitochondrial petite genomes (ρ^-) was supported. A carboxyl-terminally truncated Rpo41p exhibited a comparable activity, in spite of the fact that it was found to be transcriptionally inactive. Finally, we tested the carboxyl-terminal domain for complementation *in trans*. For this purpose the last 377 amino acid residues of yeast mitochondrial Rpo41p were fused to its mitochondrial import sequence. Coexpression of this fusion protein with C-terminally truncated Rpo41p complemented the $\Delta rpo41$ defect. These data reveal the importance of the carboxyl-terminal extension of Rpo41p for stable maintenance of intact mitochondrial genomes and for distinct species-specific intramolecular protein–protein interactions.

INTRODUCTION

In nearly all eukaryotic cells the core enzyme for mitochondrial RNA polymerase is a single-subunit protein homologous to those of bacteriophages (Cermakian *et al.*, 1996; Hedtke *et al.*, 1997; Gray and Lang, 1998). The evolutionary relation to bacteriophage RNA polymerases is evident, but the origin of these enzymes remains so far unclear (Cermakian *et al.*, 1997; Lang *et al.*, 1997). The single-subunit enzymes probably represent very ancient RNA polymerases that therefore exhibit no homologies to the eukaryotic multisubunit RNA polymerases of the nucleus (Sousa, 1996),

despite of a comparable enzyme mechanism for transcription (Delarue *et al.*, 1990; Sousa, 1996; Temiakov *et al.*, 2000).

The mitochondrial enzymes are encoded in the nucleus, transcribed in the cytosol, and imported into the organelles (Greenleaf *et al.*, 1986; Masters *et al.*, 1987; Gray and Lang, 1998). Lately, bacteriophage-type single-subunit mitochondrial core enzymes have been characterized from humans (Tiranti *et al.*, 1997) to higher plants (Hedtke *et al.*, 1997; Weihe *et al.*, 1997; Young *et al.*, 1998; Chang *et al.*, 1999; Hess and Börner, 1999). In the plant *Arabidopsis thaliana* three gene copies for organellar RNA polymerases could be identified (Börner *et al.*, 1999; Hedtke *et al.*, 2000). Two of them encode proteins of either plastid or mitochondrial location (Hedtke *et al.*, 1997; Maliga, 1998), and the third encodes an enzyme that is imported into both organelles (Hedtke *et al.*, 2000).

The single-subunit SP6/T7-type RNA polymerases of bacteriophages are characterized by a set of highly conserved domains that are supposed to be essential for catalytic functions in the process of transcription (Bonner *et al.*, 1992,

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[†] Corresponding author. E-mail address: lisowsky@uni-duesseldorf.de.

Table 1. Strains used in this study

Strain	Genotype	Source
JRY	<i>MATα</i> , <i>his4-519</i> , Δ <i>leu2</i> , <i>ura3-52</i>	J. Rine (unpublished results)
JRY	<i>MATα/MATα</i> <i>his4-519/his4-519</i> , Δ <i>leu2</i> / Δ <i>leu2</i> , <i>ura3-52/ura3-52</i>	J. Rine (unpublished results)
FY1679-18B	<i>MATα</i> , <i>his3</i> , <i>leu2</i> , <i>trp1</i> , <i>ura3</i>	
Hsrp1	<i>MATα</i> , <i>his1</i> , <i>trp2</i> , <i>ura3</i> , <i>rpo41::Tn10/URA3</i>	W.L. Fangman
DW318	<i>MATα/MATα</i> , <i>his1/HIS1</i> , <i>his3/HIS3</i> , <i>leu2/leu2</i> , <i>trp1/trp1</i> , <i>ura3/ura3</i> , <i>rpo41::Tn10/URA3/RPO41</i>	This study
DW318-7A	<i>MATα</i> , <i>his1</i> , <i>leu2</i> , <i>trp1</i> , <i>ura3</i> , <i>rpo41::Tn10/URA3</i>	This study
DW318-7D	<i>MATα</i> , <i>his3</i> , <i>leu2</i> , <i>trp1</i> , <i>ura3</i>	This study

1994a, 1994b; Sousa *et al.*, 1992, 1993; Temiakov *et al.*, 2000). Homologous domains are also present in the mitochondrial core enzymes, but these enzymes contain additional highly divergent amino- and carboxyl-terminal extensions (Masters *et al.*, 1987; Cermakian *et al.*, 1997; Hess and Börner, 1999). The yeast enzyme Rpo41p is characterized by an especially long amino-terminal extension of ~400 amino acids (Masters *et al.*, 1987). Recently, it was shown that this extension is indispensable for stable maintenance of mitochondrial genomes (Wang and Shadel, 1999). Remarkably, the amino terminal domain could complement a respective deletion mutant of Rpo41p even in *trans* (Wang and Shadel, 1999).

Today, the yeast enzyme Rpo41p represents the best model for the basic functions of mitochondrial RNA polymerases (Kelly *et al.*, 1986; Masters *et al.*, 1987; Lisowsky and Michaelis, 1989; Lisowsky *et al.*, 1996; Wang and Shadel, 1999). In contrast to the bacteriophage enzymes yeast mitochondrial RNA polymerase is dependent on the specificity factor Mtf1p (Schinkel *et al.*, 1987; Lisowsky and Michaelis, 1988; Jang and Jaehning, 1991; Shadel and Clayton, 1995; Jan *et al.*, 1999) for initiation of transcription at the conserved promoter sequences (Osinga *et al.*, 1982; Schinkel *et al.*, 1987; Xu and Clayton, 1992). In vitro the core enzyme and the specificity factor are sufficient for correct transcription of mitochondrial template DNA containing a promoter element (Schinkel *et al.*, 1987; Jang and Jaehning, 1991; Xu and Clayton, 1992). Preliminary data from higher eukaryotes indicate that organellar RNA polymerases are generally dependent on accessory factors (Diffley and Stillman, 1991; Fisher *et al.*, 1991; Parisi and Clayton, 1991; Antoshechkin and Bogenhagen, 1995; Bogenhagen, 1996; Larsson *et al.*, 1996; Börner *et al.*, 1999; Bligny *et al.*, 2000).

In this study, detailed heterologous complementation experiments with yeast and plant mitochondrial RNA polymerases were used for the first time to address the question of functional conservation of the core enzymes from different species. The well-established genetic system of yeast (Oliver, 1996; Botstein *et al.*, 1997), its defined mitochondrial transcription apparatus (Grivell, 1995), and the mitochondrial core enzyme from the plant model organism *A. thaliana* (Hedtke *et al.*, 1997) were combined in these experiments. This approach gives new insights into the function and evolution of mitochondrial core enzymes of today.

MATERIALS AND METHODS

Strains and Plasmids

A summary of the data is given in Tables 1 and 2.

Yeast strains were grown at 28 or 36°C in glucose or glycerol complete medium (2% glucose or glycerol, 1% peptone, 1% yeast extract, and appropriate amino acids or nucleotides) or minimal medium (2% glucose, 0.67% yeast nitrogen base, phosphate buffer, pH 6.2, and appropriate amino acids or nucleotides). For plates 0.2% agarose was added to the medium.

Escherichia coli strain DH5- α (Hanahan, 1983) was used for cloning experiments and amplification of plasmid DNA.

PCR Amplification

Fragments of *RPO41* were amplified from the cloned wild-type gene (Lisowsky *et al.*, 1996) by standard protocols (Innis *et al.*, 1990) using standard protocols (Innis *et al.*, 1990), the *Taq* polymerase kit (TaKaRa), and the primers listed in Table 3. The complete yeast mitochondrial *COXII* gene was isolated by PCR amplification with the listed primers using purified mitochondrial DNA or whole yeast cells (Sathe *et al.*, 1991) as template sources.

Preparation of Yeast Total DNA and RNA

Total DNA from yeast cells was isolated as described previously, and total RNA was prepared according to the protocol of Schmitt *et al.* (1990).

Table 2. Plasmids used in this study

Plasmid	Description and reference
pTL100	YEp351 containing the <i>ADH</i> promoter as a 260bp <i>SalI-SphI</i> fragment (Hofhaus <i>et al.</i> , 1999)
pTL200	pTL100 containing the complete gene for mitochondrial RNA polymerase from <i>Arabidopsis thaliana</i> cloned into the <i>SmaI</i> site behind the <i>ADH</i> promoter (This study)
pTS100	YEp351-ADH-C1 (This study)
pTS500	YEp351-ADH-C5 (This study)
pDW200	YEp351-ADH-C2 (This study)
pDW300	YEp351-ADH-C3 (This study)
pDW400	YEp351-ADH-C4 (This study)
pDW600	YEp351-ADH-C6 (This study)
p112A1NE	Used for cloning construct C7 and the wild-type gene for <i>RPO41</i> (Riesmeier <i>et al.</i> 1992)
pUC19	Vector for cloning and sequencing of mitochondrial DNA fragments (Yanisch-Perron <i>et al.</i> , 1985)
pGEM-T	Cloning of blunt end fragments and PCR products (Promega)

Table 3. Primers used in this study

Primer	Sequence and description
N1-RPO41 (5')	5'-GTTGATGATTCTAGAAGCGGTGCTTATGG-3' up-stream region of <i>RPO41</i>
N2-RPO41 (ATG)	5'-GATATTCACCTCTAGATGCTGAGACCGGCC-3' start codon of <i>RPO41</i>
C1-RPO41- <i>Nco</i> I	5'-CTGAGGCCATGGAATTTTCGACTGCTCAATTTGAG-3' construct C1
C2-RPO41- <i>Bcl</i> II	5'-TTGTGATCAAGGTGGATATCATTATACAC-3' construct C2
C3-RPO41- <i>Xho</i> I	5'-AAACTCGAGATTGCAAGAGCATTTTGGG-3' construct C3
C4-RPO41- <i>Ngo</i> MI	5'-TAAGCCGGCAGATGTTTATGCTCATGTTG-3' construct C4
C5-RPO41- <i>Ngo</i> MI	5'-ACATCTGCCGGCTTATCGCTTGAACCAGATTCAC-3' construct C5
rRPO41(3')- <i>Sma</i> I	5'-AAACCCGGGAAAGGAAATGGCTCCGCTGCTACC-3' revers primer from 3' region
Leader-RPO41- <i>Eco</i> RI	5'-CCCGAATTCATGCTGAGACCGGCCTATAAATCGCTCGTGAAAACATCCCTGTTACAAAGACGGTT AATATCTTCAAAGGGATCTAAGTTAACGGGAGATCGTTGGTGG-3' primer encoding the leader sequence of Rpo41p for mitochondrial import (construct C7)
rRPO41(3')- <i>Bam</i> HI	5'-CCCGGATCCAAAGGAAATGGCTCCGCTGCTACC-3' revers primer from 3' region
pCOXII	5'-ATGTTAGATTTATTAAGATTA-3' primer for yeast <i>COXII</i> gene
rCOXII	5'-ATTGTTTCATTTAAATCATTCC-3' revers primer for yeast <i>COXII</i> gene

Electrophoresis, Southern blots, and Hybridization

Restricted DNA samples or PCR products were electrophoretically separated in 1.0% agarose gels. Southern blots were hybridized with a mitochondrial DNA probe specific for the *COXII* gene created by PCR and the listed primers. The petite genome *E41* served as a specific probe for the mitochondrial 21S rRNA and its gene (Sor and Fukuhara, 1982). Labeling was done by the random priming method (Roche Mannheim, Germany) and [α - 32 P]dATP. Hybridization was carried out in 60% formamide, 5 $\times$ SSPE, 5 $\times$ Denhardt's, 0.1% SDS, and 100 μ g/ml herring sperm DNA at 42°C overnight after 6 h of prehybridization.

Isolation of Mitochondria, Protein Gels, and Antibody Studies

Mitochondrial protein extracts were prepared as described previously (Pratje and Michaelis, 1977). The isolated proteins were separated in gradient 4–12% SDS polyacrylamide gels (Novex/Invitrogen, Carlsbad, CA), blotted to nitrocellulose membranes and tested with antibodies specific for Rpo41p (a kind gift of Dr. G.S. Shadel) and yeast CoxIIp. Binding of antibodies was detected by alkaline-phosphatase-conjugated secondary antibodies and chemiluminescence.

Standard Techniques

Plasmid DNA was isolated from *E. coli* by alkaline lysis and Qiagen Prep Kits. Purification, restriction enzyme digestion, and ligation and analysis of yeast genomic DNA on agarose gels were performed as described (Sambrook *et al.*, 1989). Nucleotide sequences were determined by the biochemical method of Sanger *et al.* (1977) using T7 polymerase, [α - 35 S]dATP and the appropriate primers or on a sequencing machine ABI373 (Applied Biosystems, Foster City, CA). Intact yeast cells were transformed after lithium acetate treatment according to the procedure of Gietz *et al.* (1995).

RESULTS

Replacement of Yeast Mitochondrial RNA Polymerase by the Homologous Plant Enzyme

The gene for the full-length mitochondrial core enzyme of *A. thaliana* (RpoT;1: see Figure 1) was cloned into an expression vector and transformed into a diploid yeast cell that was hetero-allelic for an intact and a deleted copy of the *RPO41* gene. The use of such hetero-allelic strains is essential because elimination of the mitochondrial core enzyme from a haploid cell immediately leads to loss of mitochondrial genomes (Greenleaf *et al.*, 1986; Kelly *et al.*, 1986). We also preferred this method to plasmid shuffling because frequent recombination between the gene constructs, especially the wild-type *RPO41* gene on the shuffling plasmid, and the chromosomal gene region was observed under these conditions. Therefore, in first experiments we used tetrad dissection to generate haploid yeast mutants for Δ *rpo41* that harbored expression plasmids with the respective gene constructs. In addition, tetrad analysis is more selective for Rpo41p-dependent maintenance of mitochondrial genomes (Fangman *et al.*, 1990), because it is known that an alternative mechanism exists that allows Rpo41p-independent replication of specific small mitochondrial DNA fragments (Fangman *et al.*, 1990; MacAlpine *et al.*, 2001). Our results revealed that Δ *rpo41* mutant cells were not able to grow on nonfermentable carbon sources (see Figure 2) after transformation with the expression plasmid for the full-length mitochondrial core enzyme from *A. thaliana*. This indicated that the plant core enzyme was not able to restore normal mitochondrial transcription and the respiratory competence of these cells.

Figure 1. Constructs (C1–C7) for expression of yeast (Rpo41p) and plant (RpoT;1) mitochondrial RNA polymerases in the yeast mutant $\Delta rpo41$. Yeast Rpo41p contains at least nine (I–IX) highly conserved domains (black boxes) that are also present in the plant enzyme from *Arabidopsis thaliana* (RpoT;1). According to the latest literature some of these domains are further divided into subdomains (Cermakian *et al.*, 1997; Hess and Börner, 1999). Gray bars, plant sequences; white bars, yeast sequences. Restriction sites for exchange of domains and for cloning into yeast expression plasmids are listed. Numbers indicate the first and last amino acid residues of the protein fragments. The first 26 amino acid residues (white box) of Rpo41p are sufficient for efficient import of the protein into yeast mitochondria.

Construction of Chimeric RNA Polymerases Harboring Plant and Yeast Domains

The result that the plant enzyme alone is not able to substitute for yeast Rpo41p initiated new experiments with a set of fusion constructs of yeast and plant mitochondrial RNA polymerases. Figure 1 shows the exchange of selected protein domains. The long amino-terminal extension of Rpo41p was always included in the fusion proteins, and only highly conserved domains of the carboxyl-terminal part were exchanged. Furthermore, great care was taken to substitute fragments of identical length at identical positions. All gene constructs were checked by DNA sequencing of the complete reading frames after cloning into yeast expression vectors. These constructs (C1–C5) were transformed into the diploid tester strain with one disrupted copy of the *RPO41* gene and investigated for complementation activity of haploid $\Delta rpo41$ mutant cells after tetrad dissection (see Figure 2). It turned out that all of these chimeric genes were unable to restore respiration in the $\Delta rpo41$ yeast mutant.

Figure 2. Complementation test for a defective mitochondrial RNA polymerase in yeast. A diploid yeast strain with a normal *RPO41* gene and a defective $\Delta rpo41$ gene were used in this study. Test for respiratory competence was done after sporulation and tetrad dissection. Haploid spores with the nuclear $\Delta rpo41$ gene (*rpo41::URA3*) were identified by growth on glucose minimal medium without uracil (glucose – uracil). Before tetrad dissection the diploid cells were transformed with yeast expression plasmids as indicated. Presence of the plant mitochondrial RNA polymerase or the respective fusion constructs (see Figure 1) is listed in the left margin. Stable replication of the expression plasmid YEp351-ADH and its derivatives was tested on glucose minimal medium without leucine (glucose – leucine). Cotransformation of cells with two different plasmids (YEp351-ADH + p112ANE1) was controlled on glucose minimal medium without leucine and tryptophan (glucose – leucine – tryptophan). Respiratory competence of haploid yeast cells was tested on glycerol complete medium. At least 10 complete tetrads were analyzed for each construct.

Investigation of Mitochondrial Genomes in Yeast $\Delta rpo41$ Mutant Cells

The missing respiratory competence of $\Delta rpo41$ mutant cells harboring the chimeric plant genes could either be explained by defective mitochondrial transcription or by a loss of intact mitochondrial genomes. To address this problem, mutant cells were analyzed for their content of functional mitochondrial DNA (ρ^+). Cells without mitochondrial DNA (ρ^-) but with an intact nuclear copy of *RPO41* were crossed with the transformed mutant cells. The resulting diploid cells were tested for respiratory competence. These test crosses never generated diploid cells that were able to grow on a nonfermentable carbon source (our unpublished results). The missing respiratory competence of these cells confirmed the absence of intact mitochondrial genomes. To check mutant

cells for the presence of mitochondrial petite genomes (ρ^-), staining with a DNA-specific dye and analysis by fluorescence microscopy were performed. Most of the mutant cells were devoid of any mitochondrial DNA (ρ^0). One important exception were mutant cells transformed with gene construct C5. In this construct a carboxyl-terminal fragment of Rpo41p was replaced by the respective plant enzyme domain. All mutant cells containing this fusion construct exhibited a mitochondrial fluorescence typical for ρ^- genomes. Petite genomes were maintained in nearly all mutants cells even after longer growth and successive inoculations into fresh glucose medium.

C-terminally Truncated Rpo41p Supports Stable Maintenance of Petite Genomes

To test whether the enzyme domain of the plant mitochondrial RNA polymerase at the carboxyl-terminus of construct C5 contributed to the maintenance of deleted mitochondrial genomes, the reading frame of this domain was eliminated from the gene construct. It turned out that truncated yeast Rpo41p comprising amino acid residues 1–974 (C6; Figure 1) was sufficient for maintenance of deleted mitochondrial DNA. To verify that replication of petite genomes was dependent on the expression of truncated mitochondrial Rpo41p, we eliminated the plasmids from the $\Delta rpo41$ mutant cells. This was accomplished by incubation in glucose complete medium and subsequent selection of cells that spontaneously had lost all their plasmids. These yeast cells rapidly lost all mitochondrial ρ^- genomes immediately after elimination of the gene construct C6 (Figure 3D).

Characterization of the Mitochondrial Petite Genomes in the Mutant Cells

Total DNA was isolated from haploid mutant yeast cell cultures directly after tetrad dissection. Southern blots of restricted DNA were hybridized with a labeled mitochondrial DNA probe specific for the *COXII* gene region (see Figure 4). This DNA probe was selected, because it contains a small but well-characterized protein-encoding gene and a functional *ori/rep* sequence element (Foury *et al.*, 1998). The *COXII* probe identified a mitochondrial DNA fragment of ~20 kb in wild-type cells and the mutant transformed with construct C6, whereas mutant $\Delta rpo41$ is devoid of any mitochondrial DNA. The same result was obtained when a gene probe specific for the mitochondrial large rRNA (21S rRNA) was used. After longer incubation of the petite genome-containing mutant cells in fresh glucose medium instability of the petite genomes was observed but never the complete loss of all mitochondrial DNA fragments (our unpublished results).

The presence of the 21S rRNA and *COXII* gene on the newly formed petite genome prompted us to look for transcripts and proteins. Northern Blots with total RNA extracts were tested with the *COXII* and 21S rRNA gene probes. Only the wild-type RNA extracts exhibited transcripts for *COXII* mRNA and 21S rRNA. Crude mitochondrial protein fractions were prepared and aliquots were analyzed in Western blots. Mitochondrial CoxIIp was only present in wild-type protein extracts.

Finally, the petite genomes generated in mutant cells harboring truncated Rpo41p were tested for hypersuppressiv-

Figure 3. Fluorescence analysis of yeast cells for maintenance of mitochondrial DNA. Yeast cells were stained with the DNA-specific dye DAPI. The nucleus gives the brightest signal in each cell. The presence of mitochondrial genomes is indicated by additional peripheral fluorescence signals. (A) Wild-type, (B) mutant $\Delta rpo41$, (C) mutant $\Delta rpo41$ transformed with the expression plasmid for construct C6, (D) cells from C after elimination of the yeast expression construct C6.

ity. The phenomenon of petite suppressiveness is associated with a special class of deleted mitochondrial genomes that rapidly replace mitochondrial ρ^+ DNA from wild-type cells, thereby causing a respiratory defect (Fangman *et al.*, 1989, 1990). In crosses with wild-type ρ^+ cells and $\Delta rpo41$ mutant cells harboring the mitochondrial ρ^- genomes, we determined the percentage of respiratory-deficient diploid cells. More than 99% of the generated diploid cells still were able to grow on a nonfermentable carbon source even after longer incubation and successive replica plating (our unpublished results). This finding argued against the generation of high-suppressive petite genomes in the $\Delta rpo41$ yeast strain transformed with the chimeric gene construct C6.

Tetrad Analysis and Plasmid Shuffling Show Comparable Results

The generation of haploid tester strains by sporulation avoids the problem of generating special petite genomes that are independent of Rpo41p, because this method is more stringent than plasmid shuffling (Fangman *et al.*, 1990). To test if plasmid shuffling leads to different results, the most important constructs C2, C5, and C6 were selected for new plasmid shuffling experiments and compared with the data from tetrad analysis. Wild-type Rpo41p was expressed from the vector p112ANE that contains the tryptophan marker (Riesmeier *et al.*, 1992). This plasmid was combined with the

Figure 4. Analysis of mitochondrial DNA, RNA, and protein from wild type, the $\Delta rpo41$ mutant and the $\Delta rpo41$ mutant transformed with construct C6 ($\Delta rpo41$ + C6). Total DNA was isolated from yeast cells and aliquots of 10 μ g were restricted with *Bam*HI. After separation in a 1% agarose gel Southern blots were prepared and hybridized with gene probes representing the mitochondrial *COXII* and *21S rRNA* genes. These probes identified a higher molecular weight band of ~20 kb in the wild type and in the mutant transformed with C6 but not in the $\Delta rpo41$ mutant. Total RNA extracts from yeast cells were tested with the same mitochondrial gene probes (see MATERIAL AND METHODS). *COXII* transcripts and *21S rRNA* are only found in wild-type cells. For immunological studies aliquots of 20 μ g mitochondrial protein were separated by SDS-PAGE. The Western blot was tested with a polyclonal antibody specific for subunit 2 of cytochrome oxidase (CoxIIp). Only wild-type cells expressed detectable mitochondrial CoxIIp.

constructs C2, C5, or C6 in a haploid ρ^+ yeast cell with the $\Delta rpo41$ deletion in the nuclear genome. Elimination of the plasmids from the haploid cells was achieved by incubation in glucose complete medium. After elimination of the plasmid with the wild-type *RPO41* copy, the mutant cells were tested for respiratory competence and mitochondrial DNA content. It turned out that under these conditions all $\Delta rpo41$ cells were respiratory deficient. The cells containing C2, like a control without any plasmid, lost most of the mitochondrial petite genomes rapidly. To demonstrate the clear differences between Rpo41p-dependent and -independent petite genome maintenance, we designed a new PCR test with

Figure 5. Whole cell PCR amplification of the complete *COXII* gene for comparison of tetrad dissection and plasmid shuffling experiments. Amplified PCR products were analyzed in a 1% agarose gel. The presence of a mitochondrial genome fragment spanning this region is indicated by the amplification of a 800-base pair DNA fragment. Haploid yeast cells with a nuclear $\Delta rpo41$ were generated either by tetrad dissection or by plasmid shuffling as indicated. Plasmid constructs that were still retained in the cells are listed. Elimination of these plasmids in a second step is indicated (-). The approximate generation times for incubation in glucose medium after spore germination or plasmid elimination are listed. An aliquot of the yeast colonies was directly used for whole cell PCR amplification with primers for the complete reading frame of the *COXII* gene. Controls without any plasmids are: ρ^o strain (ρ^o); ρ^+ wild-type strain (WT).

whole yeast cells. The maintenance of the complete *COXII* region in the first tetrad dissection experiments prompted us to use primers for the complete gene in this test. It turned out that only in $\Delta rpo41$ mutant cells containing the constructs C5 or C6 was stable maintenance of larger petite genomes spanning the complete *COXII* gene region observed (see Figure 5). These experiments verify that the truncated Rpo41p for stable maintenance in haploid $\Delta rpo41$ cells. In these experiments no differences were observed whether using plasmid shuffling or tetrad dissection.

The Carboxyl-terminal Deletion of Rpo41p Can Be Complemented in trans

The finding that the carboxyl-terminal domain of yeast mitochondrial Rpo41p is essential for stable maintenance of intact mitochondrial genomes prompted us to test whether expression of the missing yeast protein fragment from a second plasmid could reconstitute a functional RNA polymerase inside mitochondria. To direct this protein fragment into yeast mitochondria, the mitochondrial import sequence of Rpo41, consisting of the first 26 amino acid residues of the core enzyme (Wang and Shadel, 1999), was fused to amino acids 903-1351 (C7; Figure 1). Complementation tests with any of the two plasmids alone never restored the respiratory function (our unpublished results). In contrast, coexpression of the two fragments of Rpo41p (C6; C7; Figures 1 and 2) in the mutant $\Delta rpo41$ restored normal respiratory functions at 28°C. The complementation activity of these two protein fragments was not identical to the full-length, wild-type core enzyme because a shift of these cells to higher temperature resulted in a block of respiratory function (see Figure 2). As a final control for complementation in *trans*, the plasmids from the complemented yeast mutant cells were again eliminated by longer growth in nonselective glucose complete medium. Spontaneous loss of the construct C6 and/or C7

Figure 6. Detection of RNA polymerases in isolated mitochondria of mutant cells. Protein extracts from isolated mitochondria were separated in a SDS polyacrylamide gel, and the Western blot was probed with a polyclonal antibody specific for yeast Rpo41p. Coexpression of construct C6 + C7 in the yeast mutant $\Delta rpo41$ allowed the detection of two distinct protein fragments with the expected molecular weights. After elimination of construct C6 from these cells the protein fragment with the corresponding molecular weight was no longer present. Examples for expression of a single-subunit fusion protein with domains from yeast and plant mitochondrial RNA polymerases are given for constructs C2 and C4. For detailed description of these constructs see Figure 1.

always revealed the original $\Delta rpo41$ mutant phenotype (our unpublished results).

Detection of Mitochondrial RNA Polymerase Fusion Proteins in Yeast Mitochondria

A precondition for functional complementation of mitochondrial transcription in $\Delta rpo41$ mutants is the correct cytosolic expression of the gene constructs on the plasmids and import of the fusion proteins into mitochondria. To test this, crude mitochondrial protein extracts were prepared and separated by SDS-PAGE. After transfer to nitrocellulose, the filter was tested with an antibody specific for Rpo41p (Figure 6). Proteins with the expected molecular weights were detected in the mitochondrial fractions. Mutant cells harboring the constructs C6 and/or C7 for fragments of Rpo41p expressed two distinct proteins of the expected molecular weights. These results confirm a strict correlation between elimination of the plasmids, loss of the respective Rpo41p fragments, and a block in respiratory function. Mutant cells transformed with a gene construct encoding just one fusion protein of yeast and plant enzymes expressed only one higher molecular weight protein that copurified

with mitochondria. Examples for fusion constructs C2 and C4 are included in Figure 6.

DISCUSSION

In eukaryotes, single-subunit RNA polymerases are the hallmark of mitochondrial transcription (Cermakian *et al.*, 1996; Hess and Börner, 1999). Our studies demonstrate the specific adaptations for organellar transcription and give new insights into the function of the C-terminal domains for replication of mitochondrial genomes. For the first time it is shown that a transcriptionally severely impaired Rpo41p fragment still supports replication of petite genomes and that this fragment of Rpo41p can complement *in trans*, upon coexpression with the missing protein domains. These data can be summarized in a new model for the evolution of single-subunit RNA polymerases by gene fusion events.

First, we investigated functional conservation of the core enzymes from yeast and plants by heterologous complementation experiments. The failure of the full-length plant enzyme to functionally substitute for Rpo41p *in vivo* could be explained by the high sequence divergence in the amino- and carboxyl-terminal regions of the mitochondrial enzymes from different species (Wang and Shadel, 1999). Therefore, the really surprising result was the finding that none of the chimeric constructs was able to replace Rpo41p. This is in contrast to many successful heterologous complementation experiments in yeast with diverse plant and human enzymes (Minet and Lacroute, 1990; Riesmeier *et al.*, 1992; Lisowsky *et al.*, 1995; Lange *et al.*, 2001). Especially the functional exchanges of components from the multisubunit RNA polymerases of the nucleus have already been documented even for only distantly related species like yeast and humans. (Shpakovski *et al.*, 1995).

Yeast mitochondrial single-subunit RNA polymerase exhibited a high sensitivity against any heterologous domain exchange. The substitution of just one small domain in the highly conserved central part of the protein already resulted in complete loss of respiratory function. According to previous experiments deletion of *RPO41* from yeast cells has two effects: 1) block of mitochondrial transcription and 2) loss of intact mitochondrial genomes (Greenleaf *et al.*, 1986). Neither were the exchanged plant enzyme domains able to complement for any of the effects of *RPO41* deletion, nor was the C-terminal plant fragment of construct C5 important for the maintenance of the deleted mitochondrial genomes in the $\Delta rpo41$ mutant, because deletion of the plant fragment from the Rpo41p fusion protein revealed a comparable phenotype. Defective mitochondrial respiration in these cells is direct evidence that the plant enzymes and chimeric fusion proteins are not able to restore normal mitochondrial transcription and maintenance of intact mitochondrial genomes.

Maintenance of mitochondrial ρ^- genomes in the $\Delta rpo41$ mutant was strictly dependent on the presence of truncated Rpo41p, whereas all the other chimeric yeast and plant enzymes did not promote replication of any petite genome. Analysis of the petite genome from haploid mutant cells directly after tetrad dissection identified mitochondrial DNA fragments of ~20 kb that contained the region including the genes for *COXII* and the *21S* rRNA (Foury *et al.*, 1998). It was not possible to identify any mitochondrial transcripts for *COXII* or the *21S* rRNA in the mutant and

consequently, no protein for CoxIIp was identified. The failure of truncated Rpo41p to synthesize mitochondrial transcripts reflects the essential function of the missing conserved domains VI-IX that are indispensable constituents of the catalytic core (Osumi-Davis *et al.*, 1992; Bonner *et al.*, 1992, 1994a, 1994b). This indicates that a normal transcription is not essential for replication of mitochondrial genomes. Comparable results were obtained in former experiments with temperature-sensitive yeast Mtf1p. Block of mitochondrial transcription at the restrictive temperature for mutated Mtf1p did not result in any reduction of mitochondrial DNA for several generations (Cliften *et al.*, 1997). Although, at this point of our analysis it cannot be excluded that truncated Rpo41p is still able to synthesize very small amounts of extremely short transcripts for initiation of mitochondrial DNA synthesis. In case that truncated Rpo41p would indeed be completely inactive for RNA synthesis, one can speculate that this protein fragment still supports the replication process either by interactions with protein components of the mitochondrial DNA replication machinery or by changing the conformation of *ori/rep* sequences as already suggested earlier (Schinkel and Tabak, 1989). First evidence for new protein interactions between the core enzyme and other mitochondrial proteins is the recent identification of a coupling mechanism between mitochondrial transcription and other components of the mitochondrial RNA metabolism by direct interaction of Rpo41p and Nam1p/Mtf2p in yeast mitochondria (Rodeheffer *et al.*, 2001). Future work will have to determine whether also components of the replication machinery interact with Rpo41p, but nevertheless our work demonstrates that even truncated and at least transcriptionally severely impaired Rpo41p supports maintenance of petite genomes. These newly generated petite genomes exhibited instability as already observed for $\Delta rpo41$ mutants (Lorimer *et al.*, 1995). Although, as long as truncated Rpo41p was present, mitochondrial DNA fragments were never completely lost from the mutant cells. The finding that these newly generated petite genomes do not show a hypersuppressive phenotype argues for the enzymatic inactivity of truncated Rpo41p, because latest results from the analysis of hypersuppressive petites demonstrate that this phenomenon depends on a functional Rpo41p and a high density of *ori* sequences generated by a large number of short repeats form the same *ori* (MacAlpine *et al.*, 2001). In addition, the work of MacAlpine *et al.* (2001) identified a hierarchy of mitochondrial promoters that may explain the generation of ρ^- genomes in our experiments with truncated Rpo41p. C-terminally truncated Rpo41p obviously still supports the function of some of the *ori/rep* sequences. The failure to synthesize transcripts from or to interact with all of the *ori/rep* sequences may induce loss of these mitochondrial DNA regions. In our experiments no differences were observed whether using tetrad dissection or plasmid shuffling for the respective experiments.

These data prove that the intact carboxyl-terminal extension of yeast Rpo41p is essential for maintenance and expression of intact mitochondrial genomes and cannot be functionally substituted by the homologous plant domains. How can these negative results for the heterologous complementation experiments with mitochondrial single-subunit RNA polymerases be explained? Several independent lines

of investigation point to specific intramolecular protein-protein interactions associated with the different domains of the core enzyme. Two-hybrid and mutation studies with Rpo41p and the specificity factor Mtf1p demonstrated that only the full-length core enzyme facilitated stable protein-protein interactions with Mtf1p and that multiple regions encompassing the entire length of the core enzyme are involved in these interactions (Cliften *et al.*, 1997, 2000). Additional evidence for special intramolecular protein-protein interactions between the domains of Rpo41p is derived from the complementation in *trans*. In contrast to the high sensitivity of Rpo41p to substitution of any domains by homologous plant enzyme fragments, the physical division of Rpo41p into separated but still functional protein fragments worked surprisingly well (Wang and Shadel, 1999).

Our experiments demonstrate that not only the amino-terminal extension (Wang and Shadel, 1999) but also the carboxyl-terminal domain can complement in *trans* a respectively truncated Rpo41p. Furthermore, for the first time, highly conserved domains of the supposed catalytic core of Rpo41p are shown to be able to complement in *trans*. For functional complementation the two separated protein fragments must still support close physical interactions to assemble a transcriptional active RNA polymerase inside mitochondria. Temperature sensitivity of this activity points toward a reduced stability of the protein complex formed by the two separated fragments of Rpo41p.

What could be the basis of such specific interactions between domains of the core enzyme Rpo41p? Our data indicate that complementation in *trans* by assembly of the two protein fragments into a functional complex inside mitochondria may resemble an early situation during the evolution of these RNA polymerases. We assume that mitochondrial single-subunit RNA polymerases of today are the result of several gene fusion events comparable to the well-documented example of FASI and FASII complexes for fatty acid biosynthesis (McCarthy and Hardie, 1984). In prokaryotes, a multisubunit FASI complex was found that was transformed into the large single-subunit FASII complex of eukaryotes by gene fusion events (McCarthy and Hardie, 1984; Schweizer *et al.*, 1984; Mohamed *et al.*, 1988; Schneider *et al.*, 1997). Comparable events for interacting domains of ancient multisubunit RNA polymerases can be postulated for the generation of single-subunit RNA polymerases of the bacteriophage-type. Adaptation and fine-tuning of these enzymes for mitochondrial transcription would have resulted in very specific intramolecular interactions for each species. According to this model the divergent amino- and carboxyl-terminal extensions from different species would represent different former accessory factors that have been recruited for adaptation to organellar transcription machineries (see Figure 7). Species-specific recruitment of these accessory factors would result in significant differences in organellar transcription regulation. This could also explain why the currently available sequences of mitochondrial specificity factors for the core enzymes exhibit high degrees of divergence (Bogenhagen, 1996; Jan *et al.*, 1999). Already among closely related yeast species, many sequence variations for the specificity factor Mtf1p can be observed (Carrodegua *et al.*, 1996; Jan *et al.*, 1999). Even more, the mitochondrial transcription apparatus of higher eukaryotes seems to have recruited completely different factors for regulation of tran-

Figure 7. Model for possible gene fusion events that generated yeast mitochondrial RNA polymerase of today. A hypothetical ancient multisubunit RNA polymerase was the ancestor of the bacteriophage-type enzymes. The bacteriophage enzymes represent a minimal form of the single-subunit RNA polymerase harboring 9 (I–IX) highly conserved domains. Adaptation of this enzyme for mitochondrial transcription required interactions with accessory factors that subsequently were fused with the core enzyme. After transfer of the gene into the nucleus of the eukaryotic cell, the attachment of an import sequence for mitochondria was necessary. Our new model does not represent the exact sequences and order of events but illustrates the possible principles that could have created such fusions.

scription initiation (Maliga, 1998; Börner *et al.*, 1999; Hess and Börner, 1999; Bligny *et al.*, 2000).

Our data give new insights into the adaptation of single-subunit RNA polymerases to the very specific conditions of organellar transcription. The importance of the core enzymes for the development of alternative regulatory mechanisms inside mitochondria is stressed by the newly identified coupling mechanism for mitochondrial transcription and RNA processing in yeast (Rodeheffer *et al.*, 2001). The specific interaction of the amino-terminal extension of Rpo41p with Nam1p/Mtf2p is the molecular basis for this phenomenon. Future work will have to determine the influence of gene fusion events and highly specific intramolecular protein interactions on the evolution of diverse functions associated with the core enzymes for mitochondrial and plastid transcription.

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REFERENCES

- Antoshechkin, I., and Bogenhagen, D.F. (1995). Distinct roles for two purified factors in transcription of *Xenopus laevis* mitochondrial DNA. *Mol. Cell. Biol.* 15, 7032–7042.
- Bligny, M., Courtois, F., Thaminy, S., Chang, C.C., Lagrange, T., Baruah-Wolff, J., Stern, D.B., and Lerbs-Mache, S. (2000). Regulation

of plastid rDNA transcription by interaction of CDF2 with two different RNA polymerases. *EMBO J.* 19, 1851–1860.

Bogenhagen, D.F. (1996). Interaction of *mtTFB* and *mtRNA* polymerase at core promoters for transcription of *Xenopus laevis* mtDNA. *J. Biol. Chem.* 271, 12036–12041.

Bonner, G., Patra, D., Lafer, E.M., and Sousa, R. (1992). Mutations in the T7 RNA Polymerase that support the proposal for a common polymerase active site structure. *EMBO J.* 11, 3767–3775.

Bonner, G., Lafer, E.M., and Sousa, R. (1994a). Characterization of a set of T7 Polymerase active site mutants. *J. Biol. Chem.* 269, 25120–25128.

Bonner, G., Lafer, E.M., and Sousa, R. (1994b). The thumb subdomain of T7 RNA Polymerase functions to stabilize the ternary complex during processive transcription. *J. Biol. Chem.* 269, 25129–25136.

Börner, T., Hedtke, B., Hess, W.R., Legen, J., Herrmann, R.G., and Weihe, A. (1999). Phage-type RNA polymerases in higher plants. In: *The Chloroplast: From Molecular Biology to Biotechnology*. ed. J.H. Argyroudi-Akoyunoglou, H. Senger, Amsterdam, Netherlands: Kluwer Academic Publishers, 73–78.

Botstein, D., Chervitz, S.A., and Cherry, J.M. (1997). Yeast as a model organism. *Science* 277, 1259–1260.

Carrodegua, J.A., Yun, S., Shadel, G.S., Clayton, D.A., and Bogenhagen, D.F. (1996). Functional conservation of yeast *mtTFB* despite extensive sequence divergence. *Gene Expr.* 6, 219–230.

Cermakian, N., Ikeda, T.M., Cedergren, R., and Gray, M.W. (1996). Sequences homologous to yeast mitochondrial and bacteriophage T3 and T7 RNA polymerases are widespread throughout the eukaryotic lineage. *Nucleic Acids Res.* 24, 648–654.

Cermakian, N., Ikeda, T.M., Miramontes, P., Lang, B.F., and Gray, M.W. (1997). On the evolution of the single-subunit RNA Polymerases. *J. Mol. Evol.* 45, 671–681.

Chang, C., Sheen, J., Bligny, M., Niwa, Y., Lerbs-Mache, S., and Stern, D.B. (1999). Functional analysis of two maize cDNAs encoding T7-like RNA polymerase. *Plant Cell* 11, 911–926.

Clifton, P.F., Park, J.-Y., Davis, B.P., Jang, S.-H., and Jaehning, J.A. (1997). Identification of three regions essential for interaction between a σ -like factor and core RNA polymerase. *Genes Dev.* 11, 2897–2909.

Clifton, P.F., Jang, S.-H., and Jaehning, J.A. (2000). Identifying a core RNA polymerase surface critical for interactions with the sigma-like specificity factor. *Mol. Cell. Biol.* 20, 7013–7023.

Delarue, M., Poch, O., Tordo, N., Moras, D., and Argos, P. (1990). An attempt to unify the structure of polymerases. *J. Protein Eng.* 10, 461–467.

Diffley, J.F.X., and Stillman, B. (1991). A close relative of the nuclear, chromosomal high-mobility group protein *HMG1* in yeast mitochondria. *Proc. Natl. Acad. Sci. USA* 88, 7864–7868.

Fangman, W.L., Henly, J.W., Churchill, G., and Brewer, B.J. (1989). Stable maintenance of a 35-base-pair mitochondrial genome. *Mol. Cell. Biol.* 9, 1917–1921.

Fangman, W.L., Henly, J.W., and Brewer, B.J. (1990). *RPO41*-independent maintenance of ρ^- mitochondrial DNA in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 10, 10–15.

Fisher, R.P., Lisowsky, T., Breen, G.A.M., and Clayton, D.A. (1991). A rapid, efficient method for purifying DNA-binding proteins. *J. Biol. Chem.* 266, 9153–9160.

Foury, F., Roganti, T., Lecrenier, N., and Purnell, B. (1998). The complete sequence of the mitochondrial genome of *Saccharomyces cerevisiae*. *FEBS Lett.* 440, 325–331.

- Grivell, L.A. (1995). Nucleo-mitochondrial interactions in mitochondrial gene expression. *Crit. Rev. Biochem. Mol. Biol.* 30, 121–164.
- Gietz, R.D., Schiestl, R.H., Willems, A.R., and Woods, R.A. (1995). Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. *Yeast* 11, 355–360.
- Gray, M.W., and Lang, B.F. (1998). Transcription in chloroplasts and mitochondria: a tale of two polymerases. *Trends Microbiol.* 6, 1–3.
- Greenleaf, A.L., Kelly, J.L., and Lehman, I.R. (1986). Yeast *RPO41* gene product is required for transcription and maintenance of the mitochondrial genome. *Proc. Natl. Acad. Sci. USA* 83, 3391–3394.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166, 557–580.
- Hedtke, B., Börner, T., and Weihe, A. (1997). Mitochondrial and chloroplast phage-type RNA polymerases in Arabidopsis. *Science* 277, 809–811.
- Hedtke, B., Börner, T., and Weihe, A. (2000). One RNA polymerase serving two genomes. *EMBO Reports* 1, 435–440.
- Hess, W.R., and Börner, T. (1999). Organellar RNA polymerases of higher plants. *Int. Rev. Cytol.* 190, 1–59.
- Hill, J.E., Myers, A.M., Koerner, T.J., and Tzagoloff, A. (1986). Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. *Yeast* 2, 163–167.
- Hofhaus, G., Stein, G., Polimeno, L., Francavilla, A., and Lisowsky, T. (1999). Highly divergent amino termini of the homologous human *ALR* and yeast *scERV1* gene products define species specific differences in cellular localization. *Eur. J. Cell Biol.* 78, 349–356.
- Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J. (1990). PCR Protocols—A Guide to Methods and Applications. San Diego: Academic Press.
- Jan, P.-S., Stein, T., Hehl, S., and Lisowsky, T. (1999). Expression studies and promoter analysis of the gene for mitochondrial transcription factor 1 (*MTF1*) from yeast. *Curr. Genet.* 36, 37–48.
- Jang, S.H., and Jaehning, J.A. (1991). The yeast mitochondrial RNA Polymerase specificity factor, *MTF1*, is similar to bacterial sigma factors. *J. Biol. Chem.* 266, 22671–22677.
- Kelly, J.L., Greenleaf, A.L., and Lehman, I.R. (1986). Isolation of the nuclear gene coding a subunit of the yeast mitochondrial RNA Polymerase. *J. Biol. Chem.* 261, 10348–10351.
- Lang, B.F., Burger, G., O’Kelly, C.J., Cedergren, R., Golding, B.G., Lemieux, C., Sankoff, D., Turmel, M., and Gray, M.W. (1997). An ancestral mitochondrial DNA resembling a eubacterial genome in miniature. *Nature* 387, 493–497.
- Lange, H., Lisowsky, T., Gerber, J., Mühlenhoff, U., Kispal, G., and Lill, R. (2001). Essential function of the mitochondrial sulphhydryl oxidase Erv1p/Alrp in the maturation of cytosolic Fe/S proteins. *EMBO Reports* 2, 715–720.
- Lisowsky, T., and Michaelis, G. (1988). A nuclear gene essential for mitochondrial replication suppresses a defect of mitochondrial transcription in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* 214, 218–223.
- Lisowsky, T., and Michaelis, G. (1989). Mutations in the genes for mitochondrial RNA polymerase and a second mitochondrial transcription factor of *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* 219, 125–128.
- Lisowsky, T., Weinstat-Saslow, D.L., Barton, N., Reeders, S.T., and Schneider, M.C. (1995). A new human gene located in the *PKD1* region of chromosome 16 is a functional homologue to *ERV1* of yeast. *Genomics* 29, 690–697.
- Lisowsky, T., Stein, T., Michaelis, G., Guan, M.-X., Chen, X.J., and Clark-Walker, D.G. (1996). A new point mutation in the nuclear gene of yeast mitochondrial RNA polymerase *RPO41* identifies a functionally important amino acid residue in a protein region conserved among mitochondrial core enzymes. *Curr. Genet.* 30, 389–395.
- Lorimer, H.E., Brewer, B.J., and Fangman, W.L. (1995). A test of the transcription model for biased inheritance of yeast mitochondrial DNA. *Mol. Cell. Biol.* 15, 4803–4809.
- MacAlpine, D.M., Kolesar, J., Okamoto, K., Butow, R.A., and Perlman, P.S. (2001). Replication and preferential inheritance of hyper-suppressive petite mitochondrial DNA. *EMBO J.* 20, 1807–1817.
- Masters, B.S., Stohl, L.L., and Clayton, D.A. (1987). Yeast mitochondrial RNA polymerase is homologous to those encoded by bacteriophages T3 and T7. *Cell* 51, 89–99.
- Maliga, P. (1998). Two plastid RNA Polymerases of higher plants: an evolving story. *Trends Plant Sci.* 3, 4–6.
- McCarthy, A.D., and Hardie, D.G. (1984). Fatty acid synthase—an example of protein evolution by gene fusion. *Trends Biochem. Sci.* 9, 60–62.
- Minet, M., and Lacroute, F. (1990). Cloning and sequencing of a human cDNA coding for a multifunctional polypeptide of the purine pathway by complementation of the *ade2-101* mutant in *Saccharomyces cerevisiae*. *Curr. Genet.* 18, 287–291.
- Oliver, S.G. (1996). From DNA sequence to biological function. *Nature* 379, 597–599.
- Osinga, K.A., De Haan, M., Christianson, T., and Tabak, H.F. (1982). A nonanucleotide sequence involved in promotion of ribosomal RNA synthesis and RNA priming of DNA replication in yeast mitochondria. *Nucleic Acids Res.* 10, 7993–8006.
- Osumi-Davis, P.A., de Aguilera, M.C., Woody, R.W., and Moon-Woody, A. (1992). Asp537, Asp812 are essential and Lys631, His811 are catalytically significant in bacteriophage T7 RNA Polymerase activity. *J. Mol. Biol.* 226, 37–45.
- Parisi, M.A., and Clayton, D.A. (1991). Similarity of human mitochondrial transcription factor 1 to high mobility group proteins. *Science* 252, 965–969.
- Pratje, E., and Michaelis, G. (1977). Allelism studies of mitochondrial mutants resistant to antimycin A or fusiculosin in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* 152, 167–174.
- Riesmeier, J.W., Willmitzer, L., and Frommer, W.B. (1992). Isolation and characterization of a sucrose carrier cDNA from spinach by functional expression in yeast. *EMBO J.* 11, 4705–4713.
- Rodeheffer, M.S., Boone, B.E., Bryan, A.C., and Shadel, G.S. (2001). Nam1p, a protein involved in RNA processing and translation, is coupled to transcription through an interaction with yeast mitochondrial RNA polymerase. *J. Biol. Chem.* 276, 8616–8622.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74, 5463–5467.
- Sathe, G.M., O’Brien, S., McLaughlin, M.M., Watson, F., and Livi, G.P. (1991). Use of polymerase chain reaction for rapid detection of gene insertions in whole yeast cells. *Nucleic Acids Res.* 19, 4775.
- Schinkel, A.H., Groot Koerkamp, M.J.A., Touw, E.P.S., and Tabak, H.F. (1987). Specificity factor of yeast mitochondrial RNA Polymerase: purification and interaction with core RNA Polymerase. *J. Biol. Chem.* 262, 12785–12791.
- Schinkel, A.H., and Tabak, H.F. (1989). Mitochondrial RNA Polymerase: dual role in transcription and replication. *Trends Genet.* 5, 149–154.

- Schmitt, M.E., Brown, T.A., and Trumpower, B.L. (1990). A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. *Nucleic Acids Res.* 18, 3091–3192.
- Schneider, R., Brors, B., Massow, M., and Weiss, H. (1997). Mitochondrial fatty acid synthesis: a relict of endosymbiotic origin and a specialized means for respiration. *FEBS Lett.* 407, 249–252.
- Schweizer, M., Lebert, C., Höltke, J., Roberts, L.M., and Schweizer, E. (1984). Molecular cloning of the yeast fatty acid synthase genes, FAS1 and FAS2: illustrating the structure of the FAS1 cluster gene by transcript mapping and transformation studies. *Mol. Gen. Genet.* 194, 457–465.
- Shadel, G.S., and Clayton, D.A. (1995). A *Saccharomyces cerevisiae* mitochondrial transcription factor. sc-mtTFB, shares features with sigma factors but is functionally distinct. *Mol. Cell. Biol.* 15, 2101–2108.
- Shpakovski, G.V., Acker, J., Wintzerith, M., Lacroix, J.-F., Thuriaux, P., and Vigneron, M. (1995). Four subunits that are shared by the three classes of RNA Polymerase are functionally interchangeable between *Homo sapiens* and *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 15, 4702–4710.
- Sor, F., and Fukuhara, H. (1982). Identification of two erythromycin resistance mutations in the gene coding for the large ribosomal RNA in yeast. *Nucleic Acids Res.* 10, 6571–6577.
- Sousa, R., Patra, D., and Lafer, E.M. (1992). Model for the mechanism of bacteriophage T7 RNAP transcription initiation and termination. *J. Mol. Biol.* 224, 319–334.
- Sousa, R., Chung, Y.J., Rose, J.P., and Wang, B.C. (1993). Crystal structure of bacteriophage T7 RNA Polymerase at 3.3Å resolution. *Nature* 364, 593–599.
- Sousa, R. (1996). Structural and mechanistic relationships between nucleic acid polymerases. *Trends Biol. Sci.* 21, 186–190.
- Temiaikov, D., Montesana, P.E., Ma, K., Mustaev, A., Borukhov, S., and McAllister, W.T. (2000). The specificity loop of T7 RNA polymerase interacts first with the promoter and then with the elongating transcript, suggesting a mechanism for promoter clearance. *Proc. Natl. Acad. Sci. USA* 97, 14109–14114.
- Tiranti, V., Savoia, A., Forti, F., Dapolito, M.F., Centra, M., Racchi, M., and Zeviani, M. (1997). Identification of the gene encoding the human mitochondrial RNA polymerase (h-mtRPOL) by cyberscreening of the expressed sequence tags database. *Hum. Mol. Genet.* 6, 615–625.
- Wakil, S.J. (1989). Fatty acid synthase, a proficient multifunctional enzyme. *Biochemistry* 28, 4523–4530.
- Wang, Y., and Shadel, G. (1999). Stability of the mitochondrial genome requires an amino-terminal domain of yeast mitochondrial RNA polymerase. *Proc. Natl. Acad. Sci. USA* 96, 8046–8051.
- Weihe, A., Hedtke, B., and Börner, T. (1997). Cloning and characterization of a cDNA encoding a bacteriophage-type RNA Polymerase from the higher plant *Chenopodium album*. *Nucleic Acids Res.* 25, 2319–2325.
- Xu, B., and Clayton, D.A. (1992). Assignment of a yeast protein necessary for mitochondrial transcription initiation. *Nucleic Acids Res.* 20, 1053–1059.
- Yanisch-Perron, C., Vieira, J., and Messing, J. (1985). Improved M13 phages cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC vectors. *Gene* 33, 103–119.
- Young, D.A., Allen, R.L., Harvey, A.J., and Lonsdale, D.M. (1998). Characterization of a gene encoding a single-subunit bacteriophage-type RNA polymerase from maize which is alternatively spliced. *Mol. Gen. Genet.* 260, 30–37.