Identifying a Core RNA Polymerase Surface Critical for Interactions with a Sigma-Like Specificity Factor

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Cyclic interactions occurring between a core RNA polymerase (RNAP) and its initiation factors are critical for transcription initiation, but little is known about subunit interaction. In this work we have identified regions of the single-subunit yeast mitochondrial RNAP (Rpo41p) important for interaction with its sigma-like specificity factor (Mtf1p). Previously we found that the whole folded structure of both polypeptides as well as specific amino acids in at least three regions of Mtf1p are required for interaction. In this work we started with an interaction-defective point mutant in Mtf1p (V135A) and used a two-hybrid selection to isolate suppressing mutations in the core polymerase. We identified suppressors in three separate regions of the RNAP which, when modeled on the structure of the closely related phage T7 RNAP, appear to lie on one surface of the protein. Additional point mutations and biochemical assays were used to confirm the importance of each region for Rpo41p-Mtf1p interactions. Remarkably, two of the three suppressors are found in regions required by T7 RNAP for DNA sequence recognition and promoter melting. Although these essential regions of the phage RNAP are poorly conserved with the mitochondrial RNAPs, they are conserved among the mitochondrial enzymes. The organellar RNAPs appear to use this surface in an alternative way for interactions with their separate sigma-like specificity factor, which, like its bacterial counterpart, provides promoter recognition and DNA melting functions to the holoenzyme.

Transcription of eukaryotic organellar genomes depends on RNA polymerases (RNAPs) distinct from the nuclear enzymes. Although the multisubunit chloroplast core RNAP is clearly related to the nuclear, eubacterial, and archaeal RNAPs, the single-subunit mitochondrial and chloroplast core RNAPs are homologous to the single-subunit RNAPs found in bacteriophages T7 and T3 (reviewed in references 6 and 9). The evolutionary relationship of the single-subunit RNAPs to the multisubunit RNAPs is uncertain, although the two classes of polymerases share many mechanistic similarities (49). The single-subunit RNAPs are actually more similar to the family of DNA polymerases and reverse transcriptases. In fact, single mutations can convert T7 RNAP to an enzyme using deoxyribonucleotide substrates (50), and conversely, a point mutation in a reverse transcriptase converts the enzyme to an RNAP (15).

For the most part, the genes that encode the single-subunit organellar RNAPs are found in the nucleus. However, in what may be a clue as to the evolutionary origin of these unusual enzymes, a single-subunit phage-like RNAP is encoded in the mitochondrial genome of a primitive brown alga (44), and genes encoding a multisubunit bacterial-like RNAP are found in the mitochondrion of an ancestral eukaryotic protozoan (29). It is therefore probable that a multisubunit bacterial-like core RNAP was replaced by a single-subunit phage-like core early in the evolution of the eukaryotic mitochondrion.

The yeast mitochondrial core RNAP, Rpo41p, is a single polypeptide of 145 kDa with striking similarity to bacterio-

phage RNAPs in the C-terminal two-thirds of the protein (33). Nine conserved regions of amino acids have been defined, including positions known to be required for structure and function of the catalytic domain (12, 25), which demonstrate especially high levels of identity. However, the N-terminal third of Rpo41p is not obviously similar to the phage RNAPs or to other proteins in the database. Although this portion of the protein is essential for function (10, 55), its role in transcription has not been elucidated.

Unlike the phage RNAPs, which function independently to recognize and bind to their promoters, open DNA at the transcription start site, and initiate transcription, Rpo41p requires a specificity factor, Mtf1p, functionally similar to bacterial sigma factors (24). Mtf1p acts like a sigma factor in that it allows the polymerase to recognize and bind to promoter DNA, and is required for proper initiation, but is then released after a short transcript has been synthesized (32). Despite these similar functions, Mtf1p has limited amino acid identity with sigma factors, although many of the shared amino acids are critical for Mtf1p function (46). In particular, we have shown that two regions of Mtf1p with similarity to sigma factors also share a functional role, contributing to interaction with the core polymerase (10). These findings suggest a structural as well as functional similarity between Mtf1p and sigma factors.

Many recent reports have focused on identifying regions and specific amino acids of sigma and sigma-like factors required for interaction with their core RNAPs (10, 26, 27, 30, 36, 47, 48, 52). From these studies it has become clear that amino acids along much of the length of sigma factor are required for core RNAP interaction. In contrast, much less is known about the regions of core RNAPs that interact with various initiation factors. Recent studies have used protein cross-linking and protein-protein interaction studies to determine interactions between sigma and the alpha, beta, and beta' subunits of the

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bacterial core RNAP (1, 11, 19, 37, 38). However, specific interaction domains are still poorly defined, due in part to the large size and complexity of the multisubunit RNAPs. The recent report of the three-dimensional structure of a bacterial core RNAP (57) in combination with the partial structure of a sigma factor (31) will certainly aid the elucidation of how all three subunits of the core RNAP interact with sigma factors.

The yeast mitochondrial core RNAP is a more tractable system for studying subunit interactions due to its modest size and polypeptide composition. Additionally, recent structural data for the T7 RNAP in the absence and presence of template (7, 25) provide an important starting point for modeling and evaluating sites of interaction identified on Rpo41p. In this work we used previously identified Mtf1p mutants defective for Rpo41p interaction to select for suppressing mutations in the core RNAP. Using the structure of T7 RNAP as a model, we have identified a surface of the mitochondrial RNAP that appears to be critical for subunit interactions. Intriguingly, the potential interaction sites on Rpo41p correspond to regions of T7 RNAP with known roles in promoter interaction. These results indicate a possible reallocation of functions from the core RNAP to the sigma-like specificity factor during the evolution of the mitochondrial RNAP.

MATERIALS AND METHODS

Media and genetic methods. Standard media such as YP medium containing 2% glucose (YPD) or 2% each glycerol, ethanol, and lactate (YPGEL), synthetic complete (SC) medium lacking the appropriate amino acid(s) and sporulation medium were prepared as described by Guthrie and Fink (20). 5-Fluoro-orotic acid (5-FOA) was added to SC medium to a final concentration of 500 mg/liter (20). *Saccharomyces cerevisiae* cells were transformed by the lithium acetate method (23). Molecular cloning techniques were as described by Sambrook et al. (45).

Two-hybrid plasmid constructs and assays. *MTF1* and *RPO41* two-hybrid clones and β -galactosidase assays were as described previously (10). Two-hybrid plasmid pVP16 was modified to facilitate cloning *RPO41* fragments for suppressor analysis and identification of subfragments responsible for suppression of *mtf1* mutants. Plasmid pVP16S (pJJ1135) was made by digesting pVP16 with *Bam*HI and ligating the *Sal*I linker GATCTGTCGACA (*Sal*I site underlined) into the overhangs. Plasmid pVP16E (pJJ1112) was made by digesting pVP16 with *Eco*RI, filling in the overhangs with Klenow fragment, and religating. Plasmid pVP16N (pJJ1113) was made by digesting pVP16 with *Nsi*I, followed by treatment with T4 DNA polymerase to remove 3' overhangs and then religation. Full-length *RPO41* was cloned into the *Not*I site of the modified vectors and shown to be fully functional in the two-hybrid assay (data not shown). The polylinker of pVP16S was sequenced to confirm proper insertion of the *Sal*I linker.

Plasmid and strain construction for the *RPO41* plasmid shuffle. A kanamycin cassette was used to disrupt *RPO41* as previously described (54). Yeast haploid strain YH58a (*MATa his4 Δ 309 ura3-52 ino1-13 leu2-3,112*) was transformed with pJJ1148 (YCplac33 [17]) containing a 5.7-kb *RPO41* fragment and then subsequently transformed with the kanamycin knockout PCR product. G418-resistant transformants were first tested by PCR to check for integration of the disruption construct in the *RPO41* gene. Properly integrated transformants were further tested to confirm that integration was in the chromosomal copy of *RPO41*. Cells were plated on 5-FOA plates to select for loss of the *RPO41*-containing plasmid. After 5-FOA treatment, the cells were plated onto YPGEL plates to determine if the cells were petite and thus disrupted for *RPO41*. A positive *rpo41 Δ* transformant containing pJJ1148 was designated yJJ1095.

RPO41 alleles were tested for function by cloning into the YCplac111 vector (17). Plasmid pJJ1149 (containing a 5.7-kb fragment of *RPO41* in YCplac111) was used. The plasmid was first modified so that the cloning vector could be distinguished from the subsequent clones. pJJ1149 was digested with *Msc*I and *Hpa*I (sites unique to the *RPO41* insert), and the fragments were religated. A mutant clone, designated pJJ1255, was recovered with the *Msc*I-*Hpa*I fragment inserted in the opposite orientation. pJJ1255 was then digested with *Bam*HI and *Nsi*I and ligated to the corresponding fragments from the two-hybrid *rpo41* mutants. In the case of I30 and I12, this fragment contains multiple mutations (see Fig. 3). The A631V and E1124K mutations were also cloned into the vector, using the *Bam*HI-*Nsi*I fragment from the two-hybrid clones.

RPO41 mutants K1273R and RQ1275AA were cloned into YCplac111 by a different strategy since these mutations lie downstream of the *Nsi*I site. The mutant two-hybrid clones were digested with *Nsi*I (producing a 1.6-kb fragment) and cloned into the *Nsi*I site of pJJ1149. This construction replaces the 3' untranslated region of *RPO41* with the pVP16 *CYC1* terminator sequence and

additional vector sequence. This change had no effect on the function of wild-type *RPO41*.

The *rpo41* mutants were tested for function in vivo using a shuffle technique. The clones were transformed into the *RPO41* disruptant strain yJJ1095 (containing wild-type *RPO41* on a *URA3* plasmid). *Leu*⁺ *Ura*⁺ transformants were plated on 5-FOA to select for the loss of the wild-type *RPO41* plasmid. *Leu*⁺ *Ura*⁻ cells were plated onto YPGEL or SCGEL-*Leu* medium to test for growth on a nonfermentable carbon source at 30 and 37°C.

PCR mutagenesis of *RPO41* for suppression analysis. To create a library of *RPO41* mutants, full-length *RPO41* was amplified by PCR using 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 50 ng of template, 30 μ M primers, 200 μ M deoxynucleoside triphosphates (dNTPs), and 5 U of *Taq* DNA polymerase per 100- μ l reaction. pJJ1137 was used as the template with primers pVP16 (5'-TGCCCTTGGAATTGACGAGTACG-3') and RPO41-3' (5'-GGATCCGCGGCGCGTTTGTAGTTCACGGCTCACGAG-3').

The PCR products from four reactions were pooled and purified using a Qiagen PCR purification column. The purified fragment was digested with *Sal*I and *Not*I and then purified by 0.8% agarose gel electrophoresis. The gel fragment was isolated using a Qiagen gel extraction column, and the purified fragment was ligated to pVP16S (pJJ1135) digested with the same enzymes. Ligation mixtures were transformed into *Escherichia coli* DH5 α cells (22). Transformants from several large transformations were pooled. The pooled cells were used to inoculate a 1-liter culture of 2 $\times$ YT medium. The cells were grown overnight, and DNA was harvested using alkaline lysis (45).

Construction of site-directed mutations in *RPO41*. *RPO41* mutants F1066I, E1124K, and K1273R identified by the suppressor analysis were recreated to separate the individual mutations and confirm that the single mutations were responsible for suppression. Additionally, we created new *RPO41* mutants using overlap extension PCR (53). Overlapping oligonucleotides containing the mutations were designed and used in separate PCRs with oligonucleotides directed to either the 5' or 3' end of *RPO41*. The oligonucleotides used are listed in Table 1. The products of the separate reactions were purified using a Qiagen PCR purification column and were then fused. The fused products were diluted 1:100 and amplified by further rounds of PCR. Cloned constructs were confirmed by digestion with the appropriate restriction enzyme.

Two-hybrid suppressor screen. *RPO41* library DNA was transformed into yeast strain L40 containing two-hybrid *MTF1* constructs in plasmid pBTM116. Cells were plated onto SC medium lacking tryptophan, uracil, leucine, and histidine (SC-THUL) supplemented with 5 mM 3-aminotriazole. The plates were incubated at either 30°C or room temperature to identify His⁺ cells. Transformants of *mtf1* mutant V135A were first plated at 37°C for 24 h and then transferred to 30°C to eliminate background growth on the minimal medium plates. His⁺ cells were rechecked by streaking the cells onto SC-THUL plates with 5 mM 3-aminotriazole. Plasmid DNA was isolated from the His⁺ cells and transformed into *E. coli* HB101 cells. The transformed cells were plated onto M9 plates containing 100 μ g each of ampicillin and proline per ml. Since the HB101 cells are *Leu*⁻, only cells complementing this defect with the yeast *LEU2* gene on the pVP16 vector can grow on this medium. Isolated plasmids were retransformed into L40 containing the *MTF1* mutant. The transformants were restreaked onto SC-THUL medium containing 3-aminotriazole to confirm that the *RPO41* plasmids were responsible for suppression.

Positive clones were sequenced at the Colorado Cancer Center DNA sequencing core facility. Additionally, the DNA regions of the clones responsible for the suppression were mapped by cloning subfragments of the suppressors into the wild-type gene. The clones were then tested for suppression of the specific *mtf1* mutants in two-hybrid strain L40. Subfragments tested included *Sal*I (vector site)-*Bam*HI, *Eco*RI-*Eco*RI, *Bam*HI-*Nsi*I, *Hpa*I-*Msc*I, *Msc*I-*Nsi*I, and *Nsi*I-*Nsi*I (vector site). The modified two-hybrid vectors described above were used to clone the suppressor subfragments.

GST-Mtf1p affinity chromatography. Interaction studies with glutathione S-transferase (GST)-Mtf1p constructs were performed as previously described (10), using morpholinepropanesulfonic acid (MOPS) buffer at pH 7.3. F3 cell extracts (56) were made from yeast strains used for in vivo testing of the *RPO41* alleles. Cells (300 ml) were grown overnight to mid-log phase in SC-*Leu* medium to maintain the *RPO41* plasmid. The cells were then diluted 1:10 in YPD medium and grown for an additional 8 to 10 h before harvesting of cells and preparation of extracts. The F3 extracts were twice dialyzed against 2 liters of M(50) (30 mM MOPS [pH 7.3], 5% glycerol, 1 mM EDTA, 10 mM MgCl₂, 50 mM KCl) for 2 h. The conductance of the extracts was less than 50 μ S cm⁻¹. Column fractions were analyzed by Western blotting as described previously (10).

Nonspecific RNAP assays. Assays were performed as described previously (56). F3 extracts were prepared as above except that the ammonium sulfate pellet was resuspended in D(0) buffer until conductance was below that of a 60 mM solution of ammonium sulfate. The amount of extract corresponding to about 1 g of yeast cells was passed over a 1-ml DEAE-cellulose (DE-52; Whatman) column. Flowthrough fractions were assayed for activity using calf thymus DNA (Sigma) as a template to determine levels of nuclear polymerase in the fractions. The fractions were then reassayed for activity on a poly(d(AT)) (Sigma) template to measure the activity of the mitochondrial polymerase.

TABLE 1. Oligonucleotides used for mutation of *RPO41*

Name	Site added or removed	Sequence (5'–3') ^a
RPO41-NotI5' RPO41-3'		CAGGCTGCGCGGCCGCGAGATGCTGAGACCGCCTATAAATC GGATCCGGCGGCCGCGTTTGTAGTTCACGGCTCACGAG
K1273Rfor K1273Rrev	<i>Bsr</i> BI	TTTGAGCGGAAAAGGCAAGAATTATTAACAGCCCT GCCTTTCCGCTCAAAATAAATTTTCATCTGCTAATGTAGT
F10066Ifor F10066Irev	<i>Ssr</i> I	CGCGAGCTCATCCATAGTGCGCATTTAATTCAGG ATGGATGAGCTCGCGGATAGCACTAAAAACATG
E1124Kfor E1124Krev	<i>Mbo</i> II	CCATATCGTAAGGAAAGCAAAAAGCAGTTGAGACC GCTTTCCTTACGATATGGCTGAACAATTGG
RQ1275AAfor RQ1275AArev	<i>Pvu</i> II	AAAAAAGCAGCTGAATTATTAACAGCCCTCTAA TAATTCAGCTGCTTTTCTCAAAATAAATTTTCATC
Y1122Afor Y1122Arev	<i>Sma</i> I	CCAGCCCGGGAAGAAAGCAAAAAGCAAG TTCTTCCCGGCTGGCTGAACAATTGGTAGTCCTAGTG
K1127Afor K1127Arev	<i>Bst</i> UI	GAAAGCGCGAAGCAAGTTGAGACCAATTTG TTGCTTCGCGCTTCTTCACGATATGGC
Q1135Afor Q1135Arev	<i>Msc</i> I	ACCAATCTGGCCACTGTCTTTATTAGCGATCC GACAGTGGCCAGATTGGTCTCAACTTGCTTTT
A633Yfor A633Yrev	<i>Bst</i> UI	GCTCCATACTTCGCGCATGGTTATCAGTAC ATGCGCGAAGTATGGAGCTTCACCGTGAAC
H636Afor H636Arev	<i>Pst</i> I	GCTTTTGCTGCAGGTTATCAGTACCACAATGG ATAACCTGCAGCAAAAGCTGGAGCTTCACC
Y638Afor Y638Arev	<i>Sac</i> I	GCGCATGGAGCTCAGTACCACAATGGTTCC CTGAGCTCCATGCGCAAAAGCTGGAGC

^a Underlining indicates the site added or removed.

RESULTS

Isolation of *RPO41* suppressors. We have previously studied interaction between Mtf1p and Rpo41p. Using two-hybrid and biochemical assays, we identified specific mutations in Mtf1p defining three distinct regions required for interaction (10) (Fig. 1A). To identify regions of Rpo41p required for interaction with Mtf1p, we applied a two-hybrid suppressor screen to select for mutations that would allow Rpo41p to interact with a defective Mtf1p mutant. A library of two-hybrid *RPO41* variants fused to the VP16 transcriptional activation domain was prepared by PCR amplification (see Materials and Methods) and screened for interaction with the interaction-defective *mtf1* mutant V135A (Fig. 1A), fused to the LexA DNA-binding domain. The V135A mutation reduces interaction with wild-type Rpo41p over 10-fold and is not functional for transcription inside the yeast mitochondrion (10). A yeast strain containing the V135A *mtf1* mutant was transformed with the *RPO41* library and plated onto His-deficient medium containing 3-aminotriazole to select for expression of the *HIS3* reporter construct. Approximately 10⁶ transformants were screened; His⁺ cells were identified and rechecked for growth on the selective medium. The *RPO41* plasmids were isolated and retransformed into the V135A *mtf1* two-hybrid strain to confirm suppression. Three suppressors of V135A were identified and initially designated I30, I12, and V1. Quantitative assays for the β -galactosidase reporter indicated that each suppressor was capable of interaction with the V135A mutation at a level about 50% of that seen between wild-type subunits (Fig. 2).

Single-point mutations confer suppression. DNA sequencing, subcloning, and site-directed mutagenesis were used to identify the mutations in *RPO41* responsible for the suppression of the V135A *mtf1* core binding mutation. Each suppressor contained multiple point mutations resulting in missense, but not nonsense, changes in the sequence (Fig. 1B), consistent with our earlier conclusions that the whole folded structure of Rpo41p is required for interaction (10). The salient mutations were identified by cloning individual DNA fragments from the suppressors back into the wild-type background (Materials and Methods) and reassaying for suppression. Subfragments determined to be responsible for suppression were resequenced to confirm that no other mutations were present.

We found that in each case suppression derived from a single amino acid substitution. As indicated in Fig. 1B, four mutations were present in the I30 mutant; the A631V substitution alone was responsible for suppression of the V135A *mtf1* mutant. For the I12 suppressor, three mutations were identified, two of which were located in the suppressing *Msc*I-*Nsi*I subfragment. Separation by site-directed mutagenesis (Materials and Methods) revealed the E1124K mutation to be solely responsible for suppression. Finally, two mutations were identified in the V1 suppressor. An *Nsi*I fragment containing the K1273R mutation as well as a part of the vector sequence was sufficient to convert the wild-type gene to a suppressor. To eliminate the possibility that vector sequences from the K1273R *Nsi*I fragment contributed to suppression, we recreated this mutation by site-directed mutagenesis. The K1273R mutant alone was capable of suppressing the V135A

FIG. 1. Features of Mtf1p and Rpo41p. (A) Comparison of Mtf1p to sigma factors and location of Mtf1p mutations that affect interactions with Rpo41p. Mtf1p mutant V135A is highlighted. Regions with sequence similarity to sigma factors are shaded (24). aas, amino acids. (B) Comparison of Rpo41p to T7 RNAP. Regions with high levels of identity are shaded. Locations of mutations in Rpo41p suppressors I30, I12, and V1 and convenient restriction enzyme sites used for subcloning the mutations are indicated. Fragments capable of converting the wild-type sequence to a suppressor are depicted as thicker lines. Mutations responsible for suppression are in boldface.

mtf1 mutation. Each of the isolated mutants exhibited the same level of interaction with the V135A mutant as the original suppressors shown in Fig. 2 (data not shown).

Allele specificity of the RPO41 suppressors. Since the three suppressors of the V135A mutation were located in three different regions of the Rpo41p sequence (Fig. 1B), it seemed unlikely that all three were identifying residues in direct contact with Mtf1p. Rather, one mutation could be in direct contact, but others could be altering the overall structure to increase binding to any *MTF1* allele. We therefore tested the

FIG. 2. Three Rpo41p constructs suppress the core-binding defect of Mtf1p mutant V135A. β -Galactosidase activity is shown for wild-type (WT) Rpo41p and the suppressor constructs interacting with Mtf1p mutant V135A. The wild-type Rpo41p interaction with wild-type Mtf1p is shown as a reference. The activity is shown for cells grown at 23°C. β -Galactosidase activity is expressed as Miller units (34).

FIG. 3. Interaction of V135A suppressors with wild-type (WT) Mtf1p (A) and Mtf1p mutant K157E (B). Cells were grown at 30°C. β -Galactosidase activity is expressed as Miller units (34).

interaction of the suppressors with wild-type and mutant forms of Mtf1p. β -Galactosidase assays indicated that interaction of the K1273R suppressor with wild-type Mtf1p was unchanged from that of wild-type Rpo41p (Fig. 3A). Therefore, the K1273R suppressor has neither increased nor decreased its affinity for wild-type Mtf1p but has simply changed in a way that selectively increases its interaction with the V135A mutation. In contrast, the A631V and E1124K suppressors both reduce interaction with wild-type Mtf1p (Fig. 3A), by 50 and 30%, respectively. These suppressors have therefore altered sites important for interaction with wild-type Mtf1p.

We found that all of the Rpo41p suppressors are relatively allele specific, in that they are unable to suppress the interaction defects of most of the noninteracting Mtf1p mutants shown in Fig. 1A (L53H, H44P, I154T, S218R, and D225G [data not shown]). However, the K1273R and E1124K Rpo41p suppressors are capable of partially correcting the temperature-sensitive defect of a nearby Mtf1p mutant, K157E (Fig. 3B). Since V135A and K157E are in the same region of Mtf1p (Fig. 1A), these mutations may have similar effects on the structure of the specificity factor which are compensated for by the E1124K and K1273R suppressors. A631V is the only completely allele specific suppressor.

Functional testing of Rpo41p mutations in vivo. The positive selection of the two-hybrid suppressor screen ensures that the folded structures of the Rpo41p suppressors are still very similar to that of the wild-type protein. Two additional points argue that the suppressors remain functional as core RNAPs. First, as outlined above, all of the mutants still have appreciable interactions with wild-type Mtf1p. Second, none of the suppressor mutations affects any of the amino acids predicted, based on similarities with T7 RNAP, to be involved in catalysis. To confirm that the suppressors could still support mitochondrial transcription in vivo, we replaced the wild-type copy of the gene in yeast with the various mutant alleles. Since func-

FIG. 4. Biochemical confirmation of interactions between the suppressors and Mtf1p mutant V135A. Whole-cell yeast extracts were prepared as described in Materials and Methods from cells expressing only wild-type (WT) or mutant forms of Rpo41p. The extracts were passed over a column of purified GST-Mtf1p that contained the V135A mutation. The columns were washed to eliminate nonspecific binding and then step eluted to release Rpo41p bound to the column. Rpo41p from input (IP), flowthrough (FT), wash, and elution fractions was detected by Western blot analysis using anti-Rpo41p antibody. The arrowheads on the left indicate a cross-reacting band (see also Fig. 7).

tional Rpo41p is required for the propagation of the mitochondrial genome (18), we constructed an *rpo41* deletion strain complemented by *RPO41* on a *URA3*-selectable vector for a plasmid shuffle replacement (Materials and Methods). *LEU2*-selectable plasmids containing the suppressor mutations were transformed into this strain, and then the cells were treated with 5-FOA to select for loss of the wild-type *RPO41 URA3* plasmid. The resulting strains were then assessed for possible defects (petite phenotype) by testing their ability to grow on a nonfermentable carbon source (glycerol-ethanol-lactate).

Consistent with the premise that the suppressors would retain catalytic function, we found that the A631V and E1124K suppressors showed no *in vivo* defect (data not shown). Apparently the partial impairment in their two-hybrid interaction with wild-type Mtf1p is not sufficient to abolish their function inside the mitochondrion. We conclude that these suppressing mutations have no significant effect on the natural function of the core mitochondrial RNAP. On the other hand, the K1273R suppressor interacted apparently normally with wild-type Mtf1p in the two-hybrid assay but has a temperature-sensitive petite phenotype *in vivo* (data not shown). Therefore, this mutation may alter the function or structure of the polymerase under certain conditions.

Biochemical interaction studies. To confirm the interaction of the Rpo41p suppressors with the V135A Mtf1p mutant, we used a biochemical assay independent of the two-hybrid fusion constructs. Using affinity chromatography, we previously demonstrated that a GST fusion construct of *mtf1* allele V135A is defective for binding wild-type Rpo41p from a yeast whole-cell transcription extract (10). To measure the ability of the Rpo41p suppressors to bind to the noninteracting V135A mutation, we prepared DNA-free transcription extracts from yeast cells expressing only the mutant forms of Rpo41p (see above). We confirmed that the V135A *mtf1* mutation was not capable of binding to wild-type Rpo41p in the transcription extract (Fig. 4). However, each of the suppressor mutations was capable of binding to the V135A fusion construct, confirming the two-hybrid results.

Site-specific mutagenesis of *RPO41*. As shown in Fig. 1B, the three suppressor mutations are located in three different regions of the Rpo41p amino acid sequence. Based on comparisons of Rpo41p to the crystal structure of the T7 RNAP (7,

25), all three suppressors should be located in unstructured or loop regions poorly conserved with the phage RNAPs. However, the A631V and E1124K suppressors are both very close to amino acids used by the phage RNAP for interactions with DNA. To confirm that these regions of the mitochondrial RNAP were in fact important for protein-protein interactions with the specificity factor, we created additional point mutations in Rpo41p near each of the suppressor mutations. The locations of the suppressor mutations in the context of predicted structural features are schematized in Fig. 5, and the site-specific mutations in these regions are described further below.

Mutations near A631V. To improve the alignment of Rpo41p to T7 RNAP in the region of the A631V suppressor, we compared it to several putative mitochondrial RNAPs and to several phage RNAPs (Fig. 5A). In this region the mitochondrial RNAPs have a somewhat conserved insertion of 16 to 20 amino acids relative to the phage RNAPs just C terminal to the sequences shown in Fig. 5A. Based on positions that appear to be conserved within the class of mitochondrial RNAPs, we created three mutations near the A631V suppressor as shown in Fig. 5A. The conserved amino acids were changed to alanine, except in one case where a conserved alanine was substituted with a tyrosine. Each mutation was tested in two-hybrid constructs and in the plasmid shuffle assay for *in vivo* function as described above. The A633Y and H636A mutations are in positions conserved in the different mitochondrial RNAPs but not in the phage RNAPs (Fig. 5A). Consistent with the fact that the A633Y mutation was completely defective for interaction with wild-type Mtf1p in the two-hybrid assay, it also resulted in a petite phenotype *in vivo* (Fig. 6). In contrast, the H636A mutation reduced the two-hybrid interaction by only 30% and was still functional *in vivo*.

The third mutation in this region, Y638A, is in a position conserved with the phage RNAPs (Fig. 5A). This mutation has no apparent defect in the two-hybrid assay but does not support transcription *in vivo* (Fig. 6). Therefore, A633 and H636 appear to identify a function unique to the mitochondrial RNAPs required for interaction between the core polymerase and the specificity factor. In contrast, the nearby position Y638 is critical for another function probably shared with the phage RNAPs.

A. A631V

B. E1124K

C. K1273R

FIG. 5. Structural and amino acid sequence features near the *RPO41* suppressor mutations. Each panel includes a schematic of structural elements flanking a suppressor mutation (position indicated by the asterisk). Cylinders and arrows denote helices and beta sheets, respectively, from the structure of T7 RNAP (25). Below each schematic is an amino acid alignment comparing the core RNAP from *S. cerevisiae* (Sc; accession no. M17539) to sequences from *N. crassa* (Nc; L25087), *S. pombe* (Sp; P13433), and *Homo sapiens* (Hs; 4826926). The boxed amino acids indicate the original suppressor; boldface indicates positions and identities of site-directed mutations described in the text. (A and B) The consensus below the mitochondrial RNAP alignments uses an uppercase letter for a four-of-four match, lowercase for a three-of-four match, and + for similar amino acids. Below the mitochondrial RNAPs is the relevant sequence from T7 RNAP (M38308) and a consensus derived from this sequence and those of phage T3 (X02981), K11 (X53238), and SP6 (Y00105). Highlighted in boldface are T7 RNAP positions involved in promoter melting or stabilization of single-stranded DNA (filled circles) and amino acids important for promoter recognition (arrowheads) as described in the text. (C) The consensus for the two fungal sequences uses uppercase for identity and + for similarity.

Mutations near E1124K. The E1124K suppressor aligns with the first amino acid of T7 RNAP in the pinky specificity loop, known to be important for interactions with the T7 RNAP promoter (42, 43). This region of the phage and mitochondrial RNAPs can be more clearly aligned than the region around the A631V suppressor, although there are significant differences between the two classes of enzymes as shown in Fig. 5B. We created three mutations in this region at sites conserved within the mitochondrial RNAPs or between the two classes of RNAPs. The Y1122A mutation lies N terminal to the specificity loop at the end of a beta sheet conserved in many polymerases, including DNA polymerases of the polymerase I fam-

FIG. 6. Analysis of site-directed Rpo41p mutations by two-hybrid and plasmid shuffle assays. Mutations were cloned into two-hybrid and yeast expression vectors as described in Materials and Methods. Two-hybrid interactions were measured for cells grown at 30°C; β -galactosidase activity is expressed as Miller units (34). The ability of the mutations to sustain growth on a nonfermentable carbon source (glycerol-ethanol-lactate) is indicated in the boxes below the bar graph. WT, wild type.

ily (25). Since the conserved beta sheet is probably required for structural integrity of the RNAP, we predicted that this mutation would result in a nonfunctional protein. Consistent with this prediction, the Y1122A mutation results in an interaction defect in the two-hybrid assay and loss of in vivo function (Fig. 6).

The second mutation within the pinky specificity loop, K1127A, was defective for two-hybrid interaction with Mtf1p but could support growth on a nonfermentable carbon source (Fig. 6). However, the strain bearing this mutation did have a large increase in petite frequency (elevated four- to sevenfold relative to wild-type Rpo41p [data not shown]), indicating that it does have significant defects in vivo. This position is not conserved between the classes of polymerases and may denote a location that contributes to interaction between the subunits but is not completely essential for this function in vivo. The third mutation was at position Q1135, which appears to be conserved within the mitochondrial RNA polymerases (Fig. 5B); however, the Q1135A mutation had no obvious defects in factor interaction or in vivo function (Fig. 6).

Mutations near K1273R. We also created a double mutation near the K1273R suppressor (Fig. 5C). This region of Rpo41p represents an insertion of amino acids relative to the phage RNAPs and all of the known mitochondrial RNAPs except that from the relatively closely related fungus *Neurospora crassa* (8). This mutation, RQ1275AA, changes two amino acids shared by Rpo41p and the *Neurospora* homologue. The double mutant reduces interaction with Mtf1p nearly fivefold (Fig. 6), supporting the idea that this insertion plays a role in subunit interaction. However, the defect is not severe enough to abrogate function since the double mutant was functional in vivo (Fig. 6).

Core RNAP assays of interaction-defective mutants. The Rpo41p suppressor mutants are clearly functional in vivo with wild-type Mtf1p as described above. In addition, some of the site-directed mutants with reduced two-hybrid interactions were found to be functional as core RNAPs in vivo (H636A, K1127A, and RQ1275AA). In contrast, site-directed mutants A633Y, Y1122A, and K1127A each reduced interaction in the two-hybrid assay and failed to support mitochondrial function in vivo. To determine if the lack of function was due strictly to

FIG. 7. Core RNAP activity of noninteracting Rpo41p mutants. As described in Materials and Methods, transcription extracts were made from yeast cells expressing wild-type (WT) Rpo41p or the indicated Rpo41p mutants. (A) Western blot of the transcription extracts with anti-Rpo41p antibody. The arrow on the left indicates the position of Rpo41p; the arrowhead on the right indicates a cross-reacting band. (B) The extracts were passed over a DEAE-cellulose column to remove nuclear RNAPs. The adsorbed fractions were then assayed to determine relative amounts of mitochondrial RNAP, using poly(d[AT]) as a nonspecific template.

a failure to interact or instead to a more serious defect precluding catalytic activity, we tested the mutant constructs in a nonselective transcription assay. Transcription extracts were prepared from the yeast strains described above expressing the mutant polymerases from a plasmid (see Materials and Methods). As shown in Fig. 7A, the wild-type and mutant proteins were present at comparable levels in all of the extracts. The extracts first were passed over a DEAE-cellulose column to remove the nuclear RNAPs (56). The DEAE-adsorbed fractions were then assayed for RNAP activity using a nonselective poly(d[AT]) template which does not require Mtf1p. Using the *rpo41* deletion strain as a control, we confirmed that essentially all of the nuclear RNAPs are removed by the DEAE column (Fig. 7B). Although the catalytic activity of the A633Y and the K1127A mutants was somewhat reduced, they still had significant transcriptional activity and are therefore correctly folded functional RNAPs. In contrast, the extract from Rpo41p mutant Y1122A, predicted to disrupt a conserved beta sheet, had no detectable catalytic activity.

DISCUSSION

Using a powerful two-hybrid suppressor screen, we have identified three regions of the yeast mitochondrial RNAP, Rpo41p, important for interactions with its sigma-like speci-

ficity factor, Mtf1p. These multiple sites, distant from each other on the linear map of the protein (Fig. 1B), support our earlier observations that essentially the entire length of the 145-kDa core RNAP is required for this interaction (10). These results are therefore consistent with the idea that the interaction surface between core RNA polymerases and initiation/specificity factors is complex. Recent work by Sharp et al. has underscored this complexity; as many as six conserved regions of bacterial sigma factors (some of which are shared with Mtf1p) are involved in interactions with the core RNAP (47). Because the structure of only a portion of sigma has been solved (31), no information on the three-dimensional arrangement of these different sites is available. The absence of a three-dimensional structure of Rpo41p precludes determination of whether the three sites we have identified are adjacent in the folded protein. However, we can use the known structure of the very similar T7 RNAP to create a working model as shown in Fig. 8.

Each of the suppressor substitutions is found in a loop region of the RNAP (Fig. 5); in some cases the mutations are in or near insertions relative to the phage RNAP. The approximate position of the suppressing amino acids shown in Fig. 8 predicts that all of the mutations may lie on one face of the RNAP (Fig. 8). This surface of the mitochondrial RNAP may therefore have diverged significantly from the phage RNAPs in both structure and function. Although all three suppressors were found to restore interaction with a single noninteracting Mtf1p mutation, it is unlikely that each of the positions indicated in Fig. 8 makes direct contact with this single amino acid. Below we present arguments for which suppressor may directly contact the V135A Mtf1p mutation and discuss the possible role of the other positions.

A631V. The A631V suppressor increases interaction with the noninteracting Mtf1p mutation V135A more than fourfold (Fig. 2). This suppressor is also highly allele specific; it reduces interaction with the wild type and does not increase interaction with other Mtf1p mutations (Fig. 3). In addition, the nearby site-directed mutation A633Y abolishes interaction with wild-type Mtf1p in the two-hybrid assay and is nonfunctional in vivo (Fig. 6), although the RNAP retains catalytic activity (Fig. 7). The predicted location of the A631V and A633Y mutations in the structure of T7 RNAP is very intriguing. As shown in Fig. 5A and 8, these residues are in a loop near the thumb domain (25). Part of this loop was unresolved in the crystal structure, but the authors speculated that it was in position to make contacts with DNA, a speculation based in part on the observation that mutations within this region abolish promoter-specific transcription without loss of catalytic activity (39, 51). This model was confirmed in the structure of T7 RNAP bound to promoter DNA (7); the loop now forms a β hairpin with valine 237 stacking on the -4 base of the template strand stabilizing the melted DNA. V237 is highlighted in the alignment shown in Fig. 5A and marked with a symbol to indicate its role in interacting with the melted DNA. Although this position is not conserved even among other phage RNAPs, the adjacent position, G238, is found in all the known phage RNAPs. Note in Fig. 5A that the sequences of the mitochondrial RNAPs differ substantially from those of the phage RNAPs in this region, indicating potentially different structures and functions. In addition to the fact that the loop is 16 to 20 amino acids longer in the mitochondrial RNA polymerases (not shown), different consensus sequences can be derived for the two classes of RNAPs. The conserved glycine at T7 position 238 is absent from the mitochondrial RNAPs; other amino acids conserved within the phage RNAPs (R231 and T244) are also missing.

Although the site of the A631V suppressor is not highly

FIG. 8. Rpo41p suppressor mutations modeled on the T7 RNAP structure. Coordinates are from reference 7. The domain colors are similar to the scheme of Jeruzalmi and Steitz (25): N-terminal domain, yellow; thumb, green; palm, red; palm insertion, orange; fingers, blue; pinky specificity loop, light blue; and extended foot, magenta. The approximate locations of the suppressor mutations (as predicted by the alignments shown in Fig. 5) are shown as starred circles on the RNAP structure and are indicated by arrows.

conserved within the mitochondrial RNAPs (Fig. 5A), it is intriguing that this suppressor mutation actually changes the yeast sequence to match that found in the enzymes from *Schizosaccharomyces pombe* and humans. It may be that this position is involved in species-specific interactions with the as yet uncharacterized specificity factors for these RNAPs. However, there is some conservation of amino acid sequences in this region of the different mitochondrial RNAPs. In particular, Rpo41p positions 632 (proline) and 633 (alanine) are relatively conserved in a broader comparison of mitochondrial RNAP sequences than that shown in Fig. 5A. Confirming the importance of this conservation, we found that site-directed mutation of the conserved alanine at 633 to tyrosine (A633Y) results in complete loss of interaction with Mtf1p (Fig. 6). Altering other apparently conserved positions in this region (H636A and Y638A) does not appreciably affect the ability of the RNAP to interact with Mtf1p; however, the Y638A mutation abolishes *in vivo* function (Fig. 6), establishing its importance for RNAP activity.

Based on these considerations, we propose that the A631V mutation defines the region of Rpo41p most likely to make direct contacts with Mtf1p position A135. As part of this speculation, we note that the added methyl group in the A631V suppressor could act to restore a hydrophobic contact lost with the Mtf1p V135A mutation. It is also possible that interaction between the subunits in this region of the mitochondrial RNAPs significantly changes the way these enzymes interact with melted DNA relative to the single-subunit phage RNAPs. This leads to the additional prediction that amino acids in Mtf1p may, like sigma factor (21), be important for melting DNA and stabilizing the open complex. This prediction is consistent with the results of Schadel and Clayton (46), who

found that some Mtf1p mutations can be corrected *in vitro* by supercoiling of template DNA.

E1124K. The E1124K suppressor increases interaction with the V135A Mtf1p mutation over fourfold, with only a slight reduction in the ability to interact with wild-type Mtf1p (Fig. 2 and 3A). E1124K also increases interaction with the noninteracting Mtf1p mutant K157E threefold (Fig. 3B). Supporting the idea that this region is important for core-factor interactions, the nearby site-directed mutation K1127A reduces interaction with wild-type Mtf1p dramatically in the two-hybrid assay (Fig. 6). Therefore suppression by E1124K is not allele specific, and although defects were detected in the two-hybrid assay, they are not severe enough to affect *in vivo* function (Fig. 6).

Like Rpo41p position V631, E1124 aligns with T7 RNAP in a region known to be involved in contacts with DNA. E1124 is the first amino acid of the pinky specificity loop (Fig. 8), an insertion within the fingers domain unique to the single-subunit RNAPs. The insertion, not found in the DNA polymerases, is thought to be a major determinant of promoter recognition by the RNAPs (49). Mutational studies initially predicted that amino acids 748 and 758 in the specificity loop of T7 RNAP would make important contacts with the -10 , -11 , and -8 positions of the promoter DNA (41, 43). These contacts were confirmed in the crystallographic studies of T7 RNAP complexed with promoter DNA (7). The crystallographic data also revealed extensive contacts between amino acids in the specificity loop and the melted template strand.

Although Rpo41p requires a specificity factor for promoter binding, the inserted pinky specificity loop is conserved in Rpo41p as well as other putative mitochondrial polymerases. However, there is little sequence conservation between the

loops of the two classes of RNAP. In Fig. 5B, the specificity loop amino acids of T7 RNAP involved in promoter recognition and single-strand DNA interactions are highlighted in boldface. The amino acids making contacts with open DNA (marked with filled circles) are conserved within the class of phage RNAPs but are not conserved with the mitochondrial RNAPs. The amino acids making promoter-specific contacts (marked with arrowheads) show some conservation within the phage enzymes, but it is the variations at these sites that are of particular interest because they determine the promoter sequence specificity of these RNAPs (41, 43). Note that there is essentially no conservation of sequence at these positions between the two classes of RNAPs. However, a consensus can be derived for the selected subset of mitochondrial RNAP sequences shown, with the greatest similarity seen at the N terminus including the end of the beta strand depicted at the top of Fig. 5B. The significance of this conservation is highlighted by our finding that the site-directed mutant Y1122A, changing a position conserved in all the RNAPs, is completely nonfunctional for interaction and catalysis (Fig. 6 and 7).

Despite the similarity seen at the end of the beta strand, there is an obvious difference: the proline at Rpo41p position 1121 (boxed in Fig. 5B). As pointed out by Cermakian et al. (6), this position is conserved in all known nucleus-encoded mitochondrial RNAPs but is not found in the phage RNAPs. This proline, which probably alters the angle of projection of the loop from the finger region, is just two amino acids from the E1124K suppressor and only six residues from the noninteracting K1127A mutation. Does this loop region in the mitochondrial RNAPs still make important contacts with DNA, or is it involved only in making protein-protein contacts with the specificity factor? Although both proteins are required for DNA binding and promoter recognition (32), there is currently no information to support a direct role for the RNAP in DNA sequence recognition. In contrast, the Mtf1p specificity factor shares some amino acid sequence similarity with region 2 of sigma factors (24), known to make promoter-specific contacts with DNA and critical for stabilization of the open promoter (reviewed in reference 21). It is therefore possible that the role of the pinky specificity loop in the mitochondrial RNAPs is to interact with Mtf1p, putting the specificity factor into the proper conformation for promoter recognition. Alternatively, the conserved proline in the mitochondrial RNAPs may alter the orientation of the loop such that it now requires interaction with the specificity factor to create the proper surface for interaction with DNA.

K1273R. The K1273R suppressor, like the E1124K suppressor, increases interaction with the V135A Mtf1p mutation nearly fourfold (Fig. 2). It does not significantly disrupt interaction with wild-type Mtf1p in the two-hybrid assay (Fig. 3A), although it does confer a ts petite phenotype in vivo. This suppressor increases interaction with the nearby K157E Mtf1p mutation two- to threefold (Fig. 3B). K1273R is located within the extended foot of the RNAP, which is an insertion relative to DNA polymerases and includes C-terminal amino acids that interact with promoter DNA and incoming rNTPs (16, 35). In Rpo41p and the *Neurospora* homologue the foot is much larger, with nearly 90 extra amino acids relative to the phage RNAPs. As shown in Fig. 5C, the region around the K1273R suppressor is quite similar between the yeast and *Neurospora* RNAPs. The site-directed double mutant RQ1275AA changes two of these shared positions and dramatically reduces interaction in the two-hybrid assay. However, the double mutant is still functional in vivo (Fig. 6), indicating that this region has a less important role in interaction than the region near the A631V suppressor.

The extra amino acids in this inserted region could either provide a surface for specificity factor interaction or serve to mask sites in Rpo41p necessary for DNA interaction, nucleotide binding, and catalysis. This second role seems unlikely since the core RNAP is active in nonspecific transcription assays with properties (including K_m for nucleotides) not unlike those of the holoenzyme (56). Since this insertion has so far been seen only in the yeast and *Neurospora* RNAPs, it raises the issue of whether all mitochondrial RNAPs have a required specificity factor. Mtf1p homologues have been identified only in other yeasts closely related to *S. cerevisiae* (5). The more distantly related mitochondrial RNAP from *Xenopus laevis* also requires a dissociable specificity factor (2), but there has been no report of a clone for this subunit to allow comparison to yeast Mtf1p.

A complex interaction surface. The suppressor screen described in this work was not exhaustive since we did not recover multiple isolates of the three identified suppressors. In addition, we previously tested a truncated form of Rpo41p lacking the N terminus, but containing all of the suppressor sites, in a two-hybrid assay and found that it was not competent for interaction (10). These facts mean that it is very likely that other regions of the Rpo41p core RNAP are also important for factor interaction. In particular, these studies do not resolve the role of the significant N-terminal extension found in the mitochondrial RNAPs relative to the phage RNAPs (Fig. 1B). Since this portion of the protein is required for interaction, either it is involved in direct contacts with the specificity factor or, like region 1 of sigma factor (13), it may be present to mask functional domains of the core RNAP made accessible after interaction.

These studies confirm the prediction that the interaction surface on bacterial RNAPs and their eukaryotic homologues is also complex. Since the cycle of interaction and dissociation of core RNAPs and their initiation factors involves many steps, this is perhaps not surprising. Core RNAPs exist in a closed conformation with the DNA binding channel relatively inaccessible (14, 57). Association with initiation factors leads to conformational changes in both components (3, 4, 19, 28) and an apparent opening of the DNA binding channel (40). The holoenzyme binds to double-stranded DNA; it then melts the DNA open and forms contacts with the fork junction and single-stranded promoter sequences (21). The RNAP initiates transcription and undergoes another conformational change resulting in escape from the promoter and dissociation from the initiation factors. Each of these steps may involve different contacts between the subunits being made and broken. The multiple contact points may therefore play different roles at defined stages of initiation. The ability to study these contacts during the transcription cycle in the relatively simple two-component mitochondrial RNAP will be very useful for understanding the complexities of the multisubunit RNAPs.

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