A Test of the Transcription Model for Biased Inheritance of Yeast Mitochondrial DNA

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Received 5 April 1995/Returned for modification 22 May 1995/Accepted 9 June 1995

Two strand-specific origins of replication appear to be required for mammalian mitochondrial DNA (mtDNA) replication. Structural equivalents of these origins are found in the *rep* sequences of *Saccharomyces cerevisiae* mtDNA. These striking similarities have contributed to a universal model for the initiation of mtDNA replication in which a primer is created by cleavage of an origin region transcript. Consistent with this model are the properties of deletion mutants of yeast mtDNA ($[rho^-]$) with a high density of *reps* (HS $[rho^-]$). These mutant mtDNAs are preferentially inherited by the progeny resulting from the mating of HS $[rho^-]$ cells with cells containing wild-type mtDNA ($[rho^+]$). This bias is presumed to result from a replication advantage conferred on HS $[rho^-]$ mtDNA by the high density of *rep* sequences acting as origins. To test whether transcription is indeed required for the preferential inheritance of HS $[rho^-]$ mtDNA, we deleted the nuclear gene (*RPO41*) for the mitochondrial RNA polymerase, reducing transcripts by at least 1000-fold. Since $[rho^-]$ genomes, but not $[rho^+]$ genomes, are stable when *RPO41* is deleted, we examined matings between HS $[rho^-]$ and neutral $[rho^-]$ cells. Neutral $[rho^-]$ mtDNAs lack *rep* sequences and are not preferentially inherited in $[rho^-] \times [rho^+]$ crosses. In HS $[rho^-] \times$ neutral $[rho^-]$ matings, the HS $[rho^-]$ mtDNA was preferentially inherited whether both parents were wild type or both were deleted for *RPO41*. Thus, transcription from the *rep* promoter does not appear to be necessary for biased inheritance. Our results, and analysis of the literature, suggest that priming by transcription is not a universal mechanism for mtDNA replication initiation.

The classical view of initiation of DNA replication proposes that *trans*-acting replication proteins interact with *cis*-acting DNA origin sequences. Replication of mitochondrial DNA (mtDNA) in *Saccharomyces cerevisiae* clearly requires nuclear gene products, but the requirements for specific *cis*-acting sequences and the role of *trans*-acting proteins in replication initiation are poorly understood. Wild-type $[rho^+]$ mtDNA has three to four sequences called *reps* (reviewed in reference 14) scattered around the genome (Fig. 1). These sequences share ~80% identity over a 300-bp sequence. Each contains three 100% conserved GC-rich blocks, termed A, B, and C, and an intact promoter adjacent to block C (Fig. 2). Another set of closely related sequences contains blocks A, B, and C but lacks intact promoters; these sequences are considered to be inactive *oris* and are not designated as *reps*. By analogy with mammalian mtDNA, the promoter and the GC-rich blocks in *rep* sequences have been proposed to be part of two *cis*-acting replication origin elements that are thought to be functionally conserved from yeasts to humans (reviewed in reference 26). One element consists of block C and a promoter immediately upstream. A promoter is found upstream of a similar GC-rich sequence (CSB II) at the heavy-strand origin of human mtDNA replication. The other putative origin element is composed of a 34-bp inverted repeat formed by blocks A and B and an AT-rich region between them (Fig. 2). This inverted repeat has the potential to form a stem-loop structure analogous to the potential stem-loop of the light-strand origin of human mtDNA replication. In contrast to yeast mtDNA, the mammalian mtDNA contains only a single set of these two elements, which are separated by a large fraction of the 16-kb genome. Two observations support a role for the promoter in replica-

tion initiation. First, both the human and the yeast mitochondrial RNA-processing RNases (MRPs), which have been implicated in the processing of RNA to produce primers for mtDNA replication, cleave the *rep* transcript in vitro near block C (6, 30). Second, in vivo DNA-RNA junctions have been mapped to the vicinity of the in vitro MRP cleavage sites in mouse (7) and human (6) mtDNAs and for two different *reps* in mutant ($[rho^-]$) mtDNAs in yeast cells (3).

mtDNA replication in *S. cerevisiae* does not require *rep* elements: respiration-deficient deletion mutants of yeast mtDNA that lack *rep* or *ori* sequences are quite common. Deleted forms of mtDNAs usually retain less than 10 kb of the wild-type 80-kb mtDNA sequences (reviewed in reference 14), and the retained fragment ($[rho^-]$ mtDNA) is amplified as a heterogeneous population of direct repeats to a mass per cell that is equivalent to that of wild-type ($[rho^+]$) mtDNA. $[rho^-]$ genomes can consist of only AT base pairs (10), with repeats as small as 35 bp (12). Thus, *rep* sequences are not required for replication of $[rho^-]$ mtDNA. Sequence requirements for mtDNA replication may be quite simple, and it is likely therefore that a large number of potential, non-*rep* replication origins exist. These non-*rep* origins may or may not be used in $[rho^+]$ mtDNA replication.

A $[rho^-]$ mitochondrial genome that contains a *rep* sequence is preferentially inherited in matings (5, 9). Matings between $[rho^+]$ haploids do not produce such an inheritance bias. When two strains with different genetically marked $[rho^+]$ mtDNAs are mated, individual cells in a resulting zygotic colony are homoplasmic, containing one or the other of the input $[rho^+]$ genomes. Most colonies contain a mixture of both types of homoplasmic cells; few colonies contain cells of only one type. In contrast, matings between $[rho^+]$ cells and $[rho^-]$ cells whose mitochondrial genomes contain *rep* sequences produce primarily zygotic colonies that have only $[rho^-]$ cells. These $[rho^-]$ mtDNAs are said to suppress the transmission of $[rho^+]$ mitochondrial genomes to diploid progeny. When

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FIG. 1. Map of $[\rho^+]$ mtDNA and $[\rho^-]$ deletion mutants. The $[\rho^+]$ mtDNA is depicted as a circle. A few mitochondrial gene landmarks are shown as filled areas. Arrowheads represent location and polarity (pointing in direction from GC element C to element A) of *ori* sequences (circled numbers 1 to 8) and *rep* sequences (rep1 to rep3 and filled arrowheads). The locations from which the $[\rho^-]$ mtDNAs used in this study originated are shown outside the circular map.

$[\rho^-]$ mtDNAs that lack *rep* sequences are mated to $[\rho^+]$ cells, the zygotic colonies are almost all $[\rho^+]$. The percentage of the total number of zygotic colonies that are completely $[\rho^-]$ is a measure of the suppressiveness. Suppressiveness is an inherent characteristic of individual $[\rho^-]$ mitochondrial genomes. When the suppressiveness of a $[\rho^-]$ strain is very high, that mtDNA is considered to be hypersuppressive (HS $[\rho^-]$). If the suppressiveness of a $[\rho^-]$ strain is less than a few percent, the mtDNA is called neutral, as the fraction of completely $[\rho^-]$ colonies is close to the fraction of colonies that would contain only one parental marker in a $[\rho^+] \times [\rho^+]$ cross. The degree of suppressiveness has been correlated with transcriptional activity for some $[\rho^-]$ genomes (2). Moreover, mutants with deletions that include the promoter and GC-rich block C show dramatically reduced inheritance bias (9).

It has been assumed, but not yet directly tested, that *rep* sequences are the preferred replication origins of the mitochondrial genome and that the preferential transmission of HS $[\rho^-]$ genomes in diploid clones from $[\rho^+] \times$ HS $[\rho^-]$ matings is due to a replication advantage caused by transcripts initiating at *rep* sequences that serve as primers for DNA synthesis (14). The nuclear gene *RPO41* encodes the only known mtRNA polymerase. If the preferential inheritance of a hypersuppressive mtDNA requires transcript synthesis and cleavage, then this property should require *RPO41*. Previous experiments in our laboratory have shown that *RPO41* is not required for mtDNA maintenance (11). This result, along with the observation that $[\rho^-]$ mtDNAs can arise from many sites in the mitochondrial genome that do not contain *rep* sequences or consensus promoters, implies that there is another mode of replication that does not require transcripts as primers. Alternatively, the role of the promoter region of the *rep* sequence in biased inheritance may be through some activity other than transcription. The published data supporting a transcript cleavage mechanism to initiate mtDNA replication are not compelling, even in mammals (see Discussion). Therefore, we have directly tested the transcription dependence of the biased inheritance of hypersuppressive $[\rho^-]$ mtDNAs by mating *RPO41* null (*rpo41Δ*) haploid cells.

FIG. 2. *rep* sequence DNA. Elements that are 100% conserved between the transcriptionally active *rep* sequences are shown in black; gray areas are less than 100% conserved. Filled arrows show the inverted repeat formed by GC elements A and B and the intervening AT sequence. The open arrow indicates the promoter and the direction of transcription.

MATERIALS AND METHODS

Strains. All *S. cerevisiae* strains used in this study were derived from A364a. The source and nuclear genotypes of the strains obtained for this study are shown in Table 1. The mitochondrial genome of SG6/82d is a spontaneous deletion derivative of the mitochondrial genome of HS $[\rho^-]$ 8-3 (27). $[\rho^0]$ variants of BS127, BS132, and 10507-5-4a were created by growing cells overnight in rich medium (YEED, which contains 1% yeast extract and 2% peptone supplemented with 2% glucose) containing 20 μ g of ethidium bromide per ml. The cells were then streaked onto YEED plates, and the subsequent colonies were screened for the absence of mtDNA by DAPI (4',6-diamidino-2-phenylindole) staining. HS3324 and HS82d mtDNAs were transferred into BS127 $[\rho^0]$ cells by cytoduction (19) via $[\rho^0]$ *kar1* mutant strains 10507-5-4a (*MATa*) and 10507-15-4b (*MATa*). N28 and M4 mtDNAs were transferred into BS132 $[\rho^0]$ cells by cytoduction with the 10507-5-4a $[\rho^0]$ *kar1* strain. Synthetic media used for selection and methods for strain manipulation have been previously described (28).

Plasmids and transformations. Cloned *RPO41* (pJJ345) was obtained from J. Jaehning. *rpo41Δ* (pUC18 containing the *rpo41Δ::URA3* fragment inserted in the polylinker at the *SalI* and *SacI* sites) was created by J. Henly in our laboratory. The *rpo41Δ* fragment consists of the 1-kb *SalI*-*BglII* 5' fragment of the *RPO41* gene ending 170 bp 5' of the ATG start codon and the 1-kb *BglII*-*SacI* 3' segment of *RPO41* containing the last 135 codons (20). The *URA3* gene with attached *BamHI* linkers replaced the *RPO41* sequences that lie between these *BglII* sites (Fig. 3). Yeast strains were transformed according to previously published methods (13). Each of the resulting strains was stained with DAPI and examined by fluorescence microscopy for the presence of mtDNA chondriolites (17). Total cellular DNA was isolated and subjected to Southern analysis to confirm the correct *RPO41* allele and mitochondrial genome for each strain.

DNA analysis. Total cellular DNA was isolated by using a modification of a previously described procedure (16). Stationary phase cells (1.5 ml) were pelleted, and then 0.3 ml of acid-washed glass beads, 0.2 ml of lysis buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA), 0.1 ml of Tris-EDTA (TE; 10 mM Tris-HCl [pH 8.0], 1 mM EDTA), 0.1 ml of phenol, and 0.1 ml of 25:1 chloroform:isoamyl alcohol were added. The mixture was vortexed for 30 s and centrifuged. The nucleic acids were precipitated from the aqueous phase by adding sodium acetate to 0.3 M and then 2.5 volumes of ethanol. RNA was eliminated by adding 500 ng of RNase A prior to restriction endonuclease digestion. The mtDNAs were probed as follows: N28 and M4 were probed with the cloned 3.7-kb *EcoRI* fragment of mitochondrial *COB* gene DNA, HS3324 was probed with cloned HS3324 DNA, and HS82d was probed with the cloned 2.0-kb *EcoRV* fragment from HS8-3 $[\rho^-]$ mtDNA.

RNA analysis. Total cellular RNA was isolated by a previously published method (22). The RNA was dissolved in 50 mM Tris-1 mM EDTA-10 mM MgCl₂-0.1 mM dithiothreitol. To reduce contaminating DNA, 2 μ g of RNase-free DNase I was added and incubated at 37°C for 1 h. The final RNA concentration was measured by UV *A*₂₆₀. For a negative control, 64 μ g of RNA from each strain was hydrolyzed with alkali according to the method of Kelly and Lehman (18). One quarter of each hydrolyzed sample and 16 μ g of DNA-free RNA were used in serial dilutions for RNA slot blots, using procedures described by Sambrook et al. (24). mtRNA was probed with the same cloned mtDNAs used in mtDNA analysis as described above; nuclear rRNA was probed with the cloned 2.85-kb *EcoRI* fragment of the 35S gene of DNA coding for rRNA.

mtDNA inheritance assay (suppressiveness). Bias in the inheritance of $[\rho^-]$ mtDNA was measured, using modifications of a previously published assay (31). Haploid cells were grown until they reached early to mid-log phase in rich medium containing 2% glucose (YEED) if the cells were $[\rho^-]$ or $[\rho^0]$ or 3% glycerol (YEPG) for $[\rho^+]$ strains. Cells (6×10^6) of each parental haploid were combined in 4 ml of nitrogen-free medium and then filtered onto sterile 24-mm-diameter 0.2- μ m-pore-size nitrocellulose filters. The filters were incubated cell side up on YEED plates for 3 h at 30°C, resuspended in nitrogen-free medium, sonicated, and plated on medium selective for diploids (synthetic medium: Trp free, His free, 10% glucose). In crosses containing a $[\rho^+]$ parent, the zygotic colonies were replica plated or streaked onto YEED and YEPG plates. Colonies that produced no significant growth on YEPG after 3 days were scored as respiration deficient. In matings lacking a $[\rho^+]$ parent, individual colonies were

TABLE 1. Yeast strains used in this study

Strain	Genotype ^a	Source
BS127 [<i>rho</i> ⁺]	<i>MATa ade1 ade2 leu2 trp1 ura3 cyh^r can^r sap^r sup^r gal⁻</i>	R. Sclafani
BS132 [<i>rho</i> ⁺]	<i>MATα ade1 ade2 his7 leu2 lys2 tyr1 ura3 CYH^s</i>	R. Sclafani
10507-5-4a [<i>rho</i> ⁺]	<i>MATa his7 kar1 lys9 ura3 can-1^r cyh-2^r</i>	P. Pryciak
10507-15-4b [<i>rho</i> ⁰]	<i>MATα aro2 his7 kar1 ura3 can-1^r cyh-2^r</i>	P. Pryciak
KL14-4a/HS3324	<i>MATa his1 leu2 trp2</i>	B. Dujon
SG6/82d	<i>MATa ade1 ade2 leu2 trp1 ura3</i>	S. Zweifel
DS400/N28	<i>MATα met</i>	A. Tzagoloff
DS400/M4	<i>MATα met</i>	A. Tzagoloff
BS127/HS3324	BS127 with HS3324 [<i>rho</i> ⁻] mtDNA	This study
BS127 <i>rpo41Δ</i> /HS3324	BS127/HS3324 with <i>rpo41Δ::URA3</i> allele	This study
BS127/HS82d	BS127 with HS82d [<i>rho</i> ⁻] mtDNA	This study
BS127 <i>rpo41Δ</i> /HS82d	BS127/HS82d with <i>rpo41Δ::URA3</i> allele	This study
BS132/N28	BS132 with N28 [<i>rho</i> ⁻] mtDNA	This study
BS132 <i>rpo41Δ</i> /N28	BS132/N28 with <i>rpo41Δ::URA3</i> allele	This study
BS132/M4	BS132 with M4 [<i>rho</i> ⁻] mtDNA	This study
BS132 <i>rpo41Δ</i> /M4	BS132/M4 with <i>rpo41Δ::URA3</i> allele	This study

^a All strains contain wild-type *RPO41* unless noted otherwise.

suspended in 5 ml of YEPD and grown overnight at 30°C. Total DNA was isolated as described above, cut with restriction endonucleases, and subjected to agarose gel electrophoresis, and the mtDNA content was determined by Southern blot analysis (29).

RESULTS

Mitochondrial protein synthesis appears to be essential for maintenance of the [*rho*⁺] genome. Conditions that eliminate mitochondrial protein synthesis, including mutations in the *RPO41* gene, result in the rapid production of [*rho*⁻] mutants (11, 21). Therefore, a test for a role for *RPO41* in mtDNA suppressiveness cannot be made by mating an HS [*rho*⁻] *rpo41Δ* strain to a [*rho*⁺] *rpo41Δ* strain. However, [*rho*⁻] mtDNAs are stably maintained in *rpo41* cells (11). When both parents have a functional *RPO41* gene, an HS [*rho*⁻] × [*rho*⁺] mating results in preferential transmission of the HS [*rho*⁻] mtDNA to the diploids, just as there is preferential transmission of [*rho*⁺] mtDNA in a [*rho*⁺] × neutral [*rho*⁻] mating. Similarly, [*rho*⁻] × [*rho*⁻] matings generally result in preferential inheritance of the [*rho*⁻] mtDNA with the higher degree of suppressiveness (23). Thus, we chose to examine the role of *RPO41* in suppressiveness, using [*rho*⁻] strains.

RNA analysis of *rpo41Δ* strains. Four [*rho*⁻] mtDNAs from three different regions of the [*rho*⁺] genome were used (Fig. 1, outside of circle). The two HS [*rho*⁻] genomes, HS3324 and HS82d, contain different *rep* sequences and consist of a 963-bp repeat and a 1.8-kb repeat, respectively. The two neutral [*rho*⁻] genomes, N28 (598-bp repeat) and M4 (3.2-kb repeat) are derived from the same region within the *COB* gene of [*rho*⁺] mtDNA and lack *rep* sequences. Unlike HS [*rho*⁻] mtDNAs, these neutral [*rho*⁻] mtDNAs do not contain mitochondrial

consensus promoters. HS3324 was previously shown to be stable in cells containing a disrupted *RPO41* allele (11). To ensure identical nuclear genetic backgrounds, each [*rho*⁻] genome was transferred by cytoduction (19) to one of two closely related haploid strains. The two neutral [*rho*⁻] mtDNAs were introduced into BS132 (*MATα*) cells that contain no mtDNA ([*rho*⁰]), and the two HS [*rho*⁻] mtDNAs were introduced into BS127 (*MATa*) [*rho*⁰] cells. *RPO41* null mutations, here called *rpo41Δ*, were introduced into isogenic strains of each mitochondrial type by recombinational replacement of the wild-type allele with a fragment of cloned DNA containing the *URA3* gene substituted for most of the *RPO41* coding region (Fig. 3). The final strains are designated by nuclear and mitochondrial genotypes. For example, BS127/HS82d has the wild-type *RPO41* allele and contains HS82d mtDNA, and BS127-*rpo41Δ*/HS82d contains the *rpo41Δ* allele and HS82d mtDNA. All four [*rho*⁻] mtDNAs were stably maintained in the *rpo41Δ* strains.

mtRNA was analyzed by slot blot hybridization to verify that mitochondrial transcription was eliminated by deletion of *RPO41*. Serial dilutions of total cellular RNA were first hybridized with ³²P-labeled mtDNA probe, the extent of hybridization was quantified, the probe was removed, and then the blots were hybridized with a nuclear DNA probe coding for rRNA to assess total RNA on the blot. In spite of the absence of a consensus promoter, the neutral [*rho*⁻] strain BS132/M4 contained substantial amounts of mitochondrial RNA, as did BS132/N28 (data not shown). Autoradiographs from a slot blot of BS132/M4 and BS132*rpo41Δ*/M4 are shown in Fig. 4. Hybridization to a probe detecting nucleus-encoded rRNA shows that similar amounts of RNA were loaded for the [*rho*⁰] and [*rho*⁻] strains (Fig. 4, right panels). RNA from [*rho*⁰] strains served as a control for the specificity of the mitochondrial probe (Fig. 4, first lane of each panel). Samples of [*rho*⁻] RNA treated with alkali before analysis did not hybridize to the probes (Fig. 4, third lane of each panel), indicating that the signals observed were due to RNA and not DNA contamination. The mtDNA probe (*COB*) hybridized to RNA from the *RPO41* M4 [*rho*⁻] strain but not to RNA from the *rpo41Δ* [*rho*⁻] strain or the [*rho*⁰] strain. Similar data were obtained with the strains containing N28, HS82d, and HS3324 mtDNA (data not shown). Quantification by phosphorimage analysis indicated that the mtRNA levels were decreased by at least 1000-fold in all of the *rpo41Δ* strains.

FIG. 3. *RPO41* deletion. Formation of the null allele of *RPO41* by a *URA3* substitution is depicted. Flanking sequence and coding region are taken from reference 20. Only relevant restriction sites are shown: B2, *Bgl*II; B1, *Bam*HI; S1, *Sal*I; Sc, *Sac*I.

FIG. 4. RNA analysis of *RPO41* and *rpo41Δ* strains. Total cellular RNAs from [*rho*⁰] cells (*rho*⁰), from BS132/M4 cells (*rho*[−]), and from BS132/M4 cells treated with alkali (alkali *rho*[−]) were analyzed by slot blot hybridization. The top panels contain RNA from *RPO41* cells, and the bottom panels contain RNA from *rpo41Δ* cells. The quantity of total RNA loaded per slot is shown in the column on the right. The blot was probed with cloned *COB* gene mtDNA to detect mtRNA (left panels) and then stripped and probed with cloned nuclear DNA coding for rRNA (rDNA) to detect nucleus-encoded RNA (right panels).

In a previous study, mitochondrial transcripts from strains containing a disrupted copy of *RPO41* were analyzed (11). The effect of the disruption on mitochondrial transcripts differed for the two mtDNAs that were compared. The hypersuppressive strain, KL14-4a/HS3324, suffered a drastic decrease in the level of mitochondrial transcripts after the *RPO41* locus was replaced with the disrupted allele. However, in the neutral [*rho*[−]] strain, KL14-4a/S4, the mitochondrial transcript levels were not significantly affected. It is possible that the disrupted *RPO41* allele produced a protein that, although incapable of specific interaction at the consensus promoter, was not completely defective. In the present studies we have used a null allele (*rpo41Δ*) instead of the disrupted allele. We found that the null allele produced no detectable mtRNA for any of the four mtDNAs tested.

Biased inheritance of *rpo41Δ* strains. The *rpo41Δ* [*rho*[−]] strains were used to determine whether transcription by *RPO41* influences hypersuppressiveness. If biased inheritance in matings results from a replication advantage conferred by transcription-driven initiation at the *rep* promoter, then the preferential transmission of the HS [*rho*[−]] mtDNA should be eliminated by deletion of *RPO41*. Alternatively, if some feature of the *rep* sequence other than transcriptional activity at the promoter is required for the preferential transmission of HS [*rho*[−]] mtDNA, the biased inheritance of HS [*rho*[−]] mtDNA should be unaffected by deletion of *RPO41*.

We first determined whether hypersuppressiveness is affected when *rpo41Δ* [*rho*[−]] strains are mated to *RPO41* [*rho*⁺] strains. As a precedent, we had shown that a recessive mutation in the *MGT1* gene eliminates the preferential transmission of HS [*rho*[−]] mtDNA when present in only one parent in HS [*rho*[−]] × [*rho*⁺] matings (31). Cells of each of the congenic

TABLE 2. Biased inheritance in [*rho*[−]] × [*rho*⁺] matings

Strain	Suppressiveness (%) ^a
<i>RPO41</i> /HS3324 ^b	94
<i>rpo41Δ</i> /HS3324 ^b	96
<i>RPO41</i> /82d ^b	100
<i>rpo41Δ</i> /82d ^b	96
<i>RPO41</i> /N28 ^c	1
<i>rpo41Δ</i> /N28 ^c	1
<i>RPO41</i> /M4 ^c	4
<i>rpo41Δ</i> /M4 ^c	3

^a Suppressiveness is measured as the percentage of diploid zygotic clones that contained only [*rho*[−]] cells, after mating BS127 or BS132 [*rho*⁺] cells to the indicated [*rho*[−]] cells. One hundred to three hundred colonies were screened for each mating.

^b BS127 nuclear background.

^c BS132 nuclear background.

rpo41Δ [*rho*[−]] strains and equivalent *RPO41* [*rho*[−]] strains were mated to [*rho*⁺] cells, and the level of suppressiveness was determined. Zygotic colonies containing only [*rho*[−]] mtDNA were detected through a screen for respiration competence, that is, by their inability to grow on medium containing a nonfermentable carbon source. Suppressiveness was unaffected by the presence of an *rpo41Δ* allele in the [*rho*[−]] haploid parent cells (Table 2). This result could mean that the level of the *RPO41* gene product brought into the zygote by the [*rho*⁺] *RPO41* parent is sufficient for the normal hypersuppressive activity.

A more stringent test of the effect of the *RPO41* null allele on biased inheritance required analysis of homozygous *rpo41Δ* zygotes. Therefore, HS [*rho*[−]] strains BS127/HS3324 and BS127/HS82d were mated, respectively, to the neutral [*rho*[−]] strains BS132/N28 and BS132/M4. In each mating, both parents contained either the wild-type *RPO41* allele or the *rpo41Δ* allele. Since growth on nonfermentable carbon sources cannot be used to measure transmission of [*rho*[−]] mtDNA in these matings, Southern analysis of restriction-digested total DNA, using probes specific for the different mtDNAs, was used to determine which mtDNA was present in a zygotic clone. For biased inheritance to be accurately measured, it is necessary to restrict the analysis to zygotes that result from matings in which both haploid parent cells contain mtDNA. If a culture of either parent used in a mating contained an appreciable fraction of [*rho*⁰] cells, the apparent transmission of mtDNA types would be affected. For example, a [*rho*⁰] cell in an HS [*rho*[−]] culture, when mated with a neutral [*rho*[−]] cell, would necessarily produce a zygotic clone containing only the neutral [*rho*[−]] mtDNA. Visual screening by fluorescent microscopy of DAPI-stained cells is not sufficiently accurate to allow precise measurement of individual [*rho*⁰] cells in an otherwise [*rho*[−]] population. Therefore, the frequency of [*rho*⁰] cells was assessed by mating cells of each [*rho*[−]] parent culture to [*rho*⁰] cells of the opposite mating type. Any [*rho*⁰] cells in the HS [*rho*[−]] or neutral [*rho*[−]] cultures will be signaled by an entire zygotic colony that, by Southern analysis, contains no mtDNA. For each [*rho*[−]] strain, no fewer than 20 of these control zygotic colonies were examined on Southern blots for the presence of mtDNA. The haploid [*rho*[−]] strains contained between 0 and 15% [*rho*⁰] cells by this measure (Table 3). In matings between different [*rho*[−]] cells, all zygotic clones examined were homoplasmic, containing either the HS [*rho*[−]] mtDNA or the

TABLE 3. Biased inheritance in HS [*rho*⁻] × neutral [*rho*⁻] matings

BS127/ <i>rho</i> ⁻ mtDNA × BS132/ <i>rho</i> ⁻ mtDNA cross	% [<i>rho</i> ⁰] cells in HS [<i>rho</i> ⁻] population	% Zygotic colonies containing:		Net suppressiveness (%)
		Neutral [<i>rho</i> ⁻] mtDNA	HS [<i>rho</i> ⁻] mtDNA	
<i>RPO41</i> /HS3324 × <i>RPO41</i> /N28 ^a	0	0	100	100
<i>rpo41Δ</i> /HS3324 × <i>rpo41Δ</i> /N28 ^a	0	0	100	100
<i>RPO41</i> /HS82d × <i>RPO41</i> /M4 ^b	5	5	95	100
<i>rpo41Δ</i> /HS82d × <i>rpo41Δ</i> /M4 ^b	15	15	85	100

^a Twenty zygotic colonies examined.
^b Forty zygotic colonies examined.

neutral [*rho*⁻] mtDNA but not significant levels of both (examples shown in Fig. 5, lanes 6 to 11). However, in these homoplasmic zygotic clones, a small amount (<1%) of the second parental [*rho*⁻] mtDNA would not have been detected. No [*rho*⁰] zygotic colonies were found, as would be expected from the low frequency of [*rho*⁰] cells in the two haploid parental populations. That is, any [*rho*⁰] cell in one of the [*rho*⁻] populations must have mated with a [*rho*⁻] cell in the other. Thus, suppressiveness was calculated as the percentage of the total zygotic clones that contained the HS [*rho*⁻] genome after the mating of two [*rho*⁻] cultures, corrected for the percent of [*rho*⁰] cells in the parental HS [*rho*⁻] culture.

In the matings between BS127/HS3324 and BS132/N28, all of the diploid colonies examined contained only HS3324 mtDNA, regardless of whether both haploid parents contained wild-type alleles (Table 3) or both contained null alleles of *RPO41* (Table 3). These haploid parent cultures had few [*rho*⁰] cells (<5%). In BS127/HS82d × BS132/M4 matings, most of the zygotic colonies contained only the hypersuppressive HS82d mtDNA, regardless of whether both parents were wild type or null for *RPO41* (Table 3). However, a few of the colonies did contain only M4 mtDNA in both the *RPO41* × *RPO41* and the *rpo41Δ* × *rpo41Δ* crosses (examples in Fig. 5, lanes 6 and 11). The percentage of cells with only M4 mtDNA (5% in the *RPO41* cross and 15% in the *rpo41Δ* cross) was exactly the same as the percentage of [*rho*⁰] cells in the two parental HS82d mtDNA strains. Thus, the actual inheritance

of the HS [*rho*⁻] mtDNA was 100% in all cases (Table 3). The number of diploid colonies screened in these experiments (20 to 40) is substantially lower than the numbers of diploid colonies examined in suppression tests in which selective growth on nonfermentable carbon sources can be used as a screen (100 to 300). Therefore, a small reduction in biased inheritance could have been missed. Nonetheless, it is clear that the mtRNA polymerase encoded by *RPO41* is not required for the strongly biased inheritance of HS [*rho*⁻] mtDNAs.

DISCUSSION

The presence of a family of highly related sequences (*rep-ori*) in *S. cerevisiae* [*rho*⁺] and suppressive [*rho*⁻] mitochondrial genomes suggests an important biological function. The correlation between the presence of active promoters in *rep* sequences and the preferential inheritance of [*rho*⁻] genomes that contain them implies a function in inheritance. One such function might be in replication initiation (5, 9). The existence of some structural and sequence homology between *rep* sequences and elements of mouse and human mtDNA replication origins supports this hypothesis and has led to a theory of a universal mechanism of mtDNA replication (26). According to this model, there are two essential origins of replication within each yeast *rep* sequence (Fig. 2). Replication starts at the first origin, which is similar to the human heavy-strand origin, from primers made by cleaving transcripts initiated at the promoter immediately upstream of GC element C. Replication of the other strand starts at a palindrome, similar to the human light-strand origin, formed by blocks A and B and the intervening AT-rich sequences. Here, initiation by a DNA primase is thought to occur after the promoter-initiated replication fork has passed it. Hypersuppressiveness, the preferential inheritance of [*rho*⁻] mtDNAs containing *rep* sequences, could then be explained by a competitive DNA replication advantage due to a greater density of these transcription-driven replication origins.

The *rep* sequence contains a consensus promoter for the *RPO41* mtRNA polymerase, and the 5' ends of transcripts have been mapped to these promoters both in vivo and in vitro (2, 25). Previous evidence suggesting an involvement of transcription in the biased transmission of HS [*rho*⁻] mtDNA was based on the fact that several HS [*rho*⁻] cells have higher transcript levels than neutral [*rho*⁻] mtDNAs which contain no *rep* sequence (reviewed in reference 4). In addition, mutations in HS [*rho*⁻] genomes that delete the promoter region were found to cause low transcript levels and very low-levels of suppressiveness. However, these mutants had also lost other sequences, including GC block C (2, 9). Thus, these observations do not prove that either the promoter or transcription per se causes hypersuppressiveness.

FIG. 5. mtDNA in zygotic colonies. Individual zygotic colonies were grown overnight in liquid medium. Total cellular DNA was isolated from 1.5 ml of stationary-phase cells, digested with restriction endonuclease *Taq* I, and then subjected to gel electrophoresis and Southern blot analysis. Representative examples of zygotic colony DNA probed with both cloned *COB* DNA and cloned HS8-3 DNA are shown. Haploid parents of the zygotes are listed above the gel. +, wild-type allele of *RPO41*; Δ, null allele of *RPO41*. Lanes 1 to 4: total DNA from one diploid colony from each parental [*rho*⁻] haploid mated with [*rho*⁰] (*rho*⁰) cells. Lane 5: [*rho*⁰] haploid DNA (*rho*⁰). Lanes 6 to 8: three independent colonies (a, b, and c) from BS127/HS82d × BS132/M4 zygotes. Lanes 9 to 11: three independent colonies (a, b, and c) from BS127/*rpo41Δ*/HS82d × BS132/*rpo41Δ*/M4 zygotes.

We have shown here that mtRNA polymerase activity is not required for hypersuppressiveness of HS [*rho*⁻] mtDNA, since cells containing the null allele of *RPO41* maintained their characteristic inheritance bias. One interpretation of our results is that *rep* sequences do not act by providing a replication advantage in the zygote but that they give a segregation advantage instead. For example, some part of the *rep* sequence, possibly including the promoter, may serve as a site for preferential attachment to the mitochondrial membrane; inheritance bias could be caused by the prevalence or scarcity of mtDNA molecules available to move into the daughter bud. Evidence suggesting an in vivo association of yeast mtDNA with mitochondrial structures has been obtained (1). Alternatively, it is possible that *rep* sequences do provide a replication advantage in the zygote but that transcription is not involved in this mechanism of replication initiation.

What is the evidence that replication initiation in yeast mitochondria is primed by transcription? Only one study of *S. cerevisiae* mtDNA replication origins in vivo has been reported. Presumed origins of bidirectional replication were mapped to the *rep* sequences of HS [*rho*⁻] mtDNAs by S1 nuclease analysis (3). The 5' ends of DNA molecules were located downstream from the promoter, and some of these DNAs were joined to RNAs. Both results are expected from the transcript cleavage model for priming mtDNA replication. However, while the 5' ends of essentially all long RNA molecules mapped to the promoter, a small fraction of RNA 5' ends, which were attached to DNA, mapped downstream from the promoter. No evidence that the 5' ends of RNAs joined to DNA mapped to the promoter was obtained. Additionally, nascent DNA from the opposite strand had RNA-capped 5' ends that mapped to the same area, not to the A-B palindrome region. These results are consistent with initiation on both strands at or near GC block C by a DNA primase. Evidence that primers for DNA synthesis could be created by cleavage of *rep* transcripts comes from in vitro studies. Purified MRP endoribonucleases (from both *S. cerevisiae* and humans) can cleave *rep* transcripts that are made in vitro from an SP6 promoter, at or about the same positions that RNA-DNA junctions were mapped in vivo (30). However, while mutational analysis has shown that the yeast MRP is required for normal processing of nuclear rRNA in vivo (8, 26a), a role for the enzyme in the mitochondrion in vivo has not yet been demonstrated for any organism.

The hypothesis that replication in yeast mtDNA is initiated by cleavage of the *rep* transcript is heavily dependent upon observations with mammalian mtDNA systems. However, the data for promoter-driven mammalian mtDNA initiation are not complete. The three major human in vivo 5' mtDNA ends do not map to the sites of in vitro MRP cleavage in human mtRNA (6). One of the in vivo ends actually maps 33 bp upstream of the promoter, and the other two map at sites more than 100 bp downstream of the MRP cleavage site. Only one of four additional minor in vivo mtDNA initiation sites maps to the MRP cleavage site. In humans, no evidence for any type of RNA primer at the major in vivo 5' mtDNA ends has been demonstrated. In mice, in vivo mtDNA strands from the D-loop region were shown to have RNA primers attached to their 5' ends (7). The 3' ends of RNA molecules that initiate in the promoter region were also mapped, and a small fraction of these 3' ends do map to the location of the 5' mtDNA ends, ~150 nucleotides downstream of the promoter region, as expected for the transcript cleavage model. However, most of the nascent DNA-RNA copolymers have very short RNA stretches that do not begin at the promoter. Thus, even in mammalian

mtDNA replication, the evidence for promoter-driven replication initiation is not yet definitive.

A requirement for *rep* sequence in the biased transmission of mtDNAs from yeast zygotes is very clear. However, there is little information about which elements within *rep* are required. An involvement of GC block A is supported by observations with two [*rho*⁻] mtDNAs in which deletions in block A reduced suppressiveness about twofold (15). This phenotype is temperature sensitive and may be the result of a thermally unstable cruciform created at the inverted repeat between GC block A and GC block B (2). While the *rep* sequences in yeast mtDNA have an intriguing resemblance to the two replication origins in mammalian mitochondrial genomes, definitive evidence for origin function and possible mechanisms of initiation will require further molecular and genetic analyses. A systematic determination of the role of *rep* sequence elements in preferential inheritance will require systematic mutational alteration and analysis. If hypersuppressiveness can be established by transforming cloned *rep* sequences into [*rho*⁰] yeast mitochondria, then functional assays of altered *rep* constructs will become possible.

ACKNOWLEDGMENTS

We thank Daniel Lockshon, Teresa Ward, and Stephan Zweifel for technical advice and comments on the manuscript. Autoradiographic analysis was carried out at the PhosphorImager Facility of the Markey Molecular Medicine Center at the University of Washington.

This work was supported by Public Health Service grant GM43847 (to W.L.F. and B.J.B.) and by American Cancer Society Postdoctoral Fellowship PF4105 (to H.E.L.).

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