Role of Transcription in Plasmid Maintenance in the *hpr1* Δ Mutant of *Saccharomyces cerevisiae*

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The *Saccharomyces cerevisiae* hyperrecombination mutation *hpr1* Δ results in instability of sequences between direct repeats that is dependent on transcription of the repeat. Here it is shown that the *HPR1* gene also functions in plasmid stability in the presence of destabilizing transcription elongation. In the *hpr1* Δ mutant, plasmid instability results from unchecked transcription elongation, which can be suppressed by a strong transcription terminator. The plasmid system has been used to examine in vivo aspects of transcription in the absence of Hpr1p. Nuclear run-on studies suggest that there is an increased RNA polymerase II density in the *hpr1* Δ mutant strain, but this is not accompanied by an increase in accumulation of cytoplasmic mRNA. Suppression of plasmid instability in *hpr1* Δ can also be achieved by high-copy expression of the RNA splicing factor *SUB2*, which has recently been proposed to function in mRNA export, in addition to its role in pre-mRNA splicing. High-copy-number *SUB2* expression is accompanied by an increase in message accumulation from the plasmid, suggesting that the mechanism of suppression by Sub2p involves the formation of mature mRNA. Models for the role of Hpr1p in mature mRNA formation and the cause of plasmid instability in the absence of the Hpr1 protein are discussed.

The *hpr1* mutant was originally identified in a genetic screen to identify mutants with increased intrachromosomal recombination events between direct repeat sequences in yeast (2). The majority of these events in the *hpr1* mutant resulted in a deletion of the intervening sequence between the direct repeats, leaving only one copy of the original repeat (1). Genetic suppressors of this phenotype cluster in mutations in components of the RNA polymerase II transcriptional machinery. *rpb2*, *sua7* (TFIIB), *sin4* (13, 14), *srb2* (29), *gcr3* (45), and *gal11* (31) all suppress *hpr1*-stimulated hyperrecombination to some degree. This finding has led to the idea that *HPR1* has a functional role in RNA polymerase II transcription. Indeed, *hpr1*-stimulated hyperrecombination has been demonstrated to be dependent on transcription (13) and specifically dependent on transcriptional elongation (7, 32).

Additionally, Hpr1p has been demonstrated to associate with two different protein complexes, each further linking *HPR1* to transcription. Hpr1p associates with Paf1p, Cdc73p, and Ccr4p as part of an RNAPII complex that is a distinct form of RNA polymerase II, characterized by the absence of Srb proteins (6). Both *cdc73* Δ and *paf1* Δ mutants display hyperrecombination phenotypes, although the phenotypes are not as strong as that of the *hpr1* Δ mutant. Consistent with the assembly of Hpr1p into this RNAPII complex, the *hpr1* Δ mutation is lethal when combined with either a *paf1* Δ or a *ccr4* Δ mutation, and the double-mutant *hpr1* Δ *cdc73* Δ strain displays temperature-sensitive growth at 30°C. Hpr1p has also been demonstrated to associate with a separate protein complex, termed

the THO complex, that contains Tho2p, Mft1p, and Thp2p (8). The exact function of this complex is not clear; however, it appears to be involved in linking transcription to mRNA export and is associated with the Yra1p, Tex1p, and Sub2p factors (41), which have been linked to mRNA export (34, 40). Importantly, *tho2* Δ , *mft1* Δ , and *thp2* Δ mutants display the hyperrecombination phenotype observed in the *hpr1* Δ mutant and have impaired transcriptional elongation through the bacterial *lacZ* sequence (8, 30). It has been proposed that the THO protein complex acts as a functional unit connecting transcription elongation with mitotic recombination.

Other genetic data suggest that *HPR1* in some way influences the structural integrity of DNA. First, a full complement of topoisomerase activities is necessary for viability in *hpr1* Δ strains (3). Second, the *hpr1* Δ mutation is lethal when combined with a deletion of histones H3 and H4 (*hht1-hhf1* Δ) (14). Consistent with these findings, overexpression of either *HHT1-HHF1* or *HTA-HTB* (H2A-H2B) results in a severe growth defect when combined with an *hpr1* Δ mutation (51). Overexpression of all four histones has no effect on the growth of an *hpr1* Δ mutant strain.

Although there is evidence that Hpr1p promotes transcriptional elongation, the exact function it performs that allows it to promote elongation is not known and its recent association with mRNA export factors complicates the picture (40, 41). Previous experiments have demonstrated that Hpr1p-deficient cells have trouble transcribing through both the bacterial *lacZ* sequence and the bacterial *ori-amp* sequence of pBR322, resulting in the production of truncated transcripts (7, 32). Transcriptional run-on experiments have also provided some evidence that transcriptional blocks may be localized to a specific region of *lacZ* (7). Importantly, when these sequences are located between direct repeats, transcriptional elongation through them is linked to the recombination events observed

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between the repeats in *hpr1Δ* mutants. If elongation can be stopped prior to entering these sequences with the use of a strong transcription terminator, the recombination phenotype is not observed (7, 32). Therefore, there appears to be a direct link in *hpr1Δ* cells between the difficulty in transcribing these sequences and the hyperrecombination phenotypes observed. However, more recent results suggest that the defective transcription of *lacZ* segments in Hpr1p-deficient cells cannot be correlated with a particular DNA sequence or structure (9). Hpr1p seems to be required for transcription of particularly long DNA sequences or G+C-rich sequences, and the transcription impairment through such sequences is correlated with their ability to stimulate direct repeat recombination. This transcription defect can be suppressed by high-copy-number Sub2p (18). Transcription impairment is associated with a decrease in the steady-state accumulation of full-length mRNA. These observations have been extended to decreased transcription of endogenous DNA sequences that are long and G+C rich and direct repeat stimulation by these same sequences in an *hpr1Δ* mutant (9). However, exactly how Hpr1p functions in transcription elongation is not understood.

We have reported that *hpr1Δ* strains have a defect in nuclear mRNA export (34). We have also observed that high-copy expression of *SUB2*, a DEAD box family RNA helicase motif-containing gene involved in prespliceosome formation (19, 22, 24, 50), suppresses *hpr1Δ*-stimulated recombination and other *hpr1Δ*-associated phenotypes (15) and that *sub2* mutants have a similar hyperrecombination phenotype that can be suppressed by high-copy-number *HPR1* (15). Interestingly, *SUB2* has recently been implicated in nuclear mRNA export of intron-containing and intronless transcripts.

Here we describe a situation in which transcriptional elongation through the bacterial *ori-amp* sequence from a yeast promoter present on a *CEN*-based plasmid contributes to its own instability in an *hpr1Δ* mutant strain. We have used the plasmid loss phenotype to gain further insight into the transcriptional defects present in *hpr1Δ* strains and have examined the effects of high-copy-number *SUB2* on the stability and transcription of the unstable plasmid in *hpr1Δ* mutant strains to learn more about the mechanism of suppression by Sub2p and the role of Hpr1p in transcription and mRNA maturation.

MATERIALS AND METHODS

Strains and growth media. The strains used in this study are listed in Table 1. All of the strains are isogenic to the W303 *RAD5⁺* background. Nonselective synthetic complete medium (SC) and SC lacking the indicated amino acids were prepared by standard methods (37). All strains were grown at 30°C. For experiments requiring 6-azauracil (6-AU; Sigma), 100 μg of 6-AU per ml was added to liquid SC lacking tryptophan and uracil (SC-Trp-Ura) after autoclaving.

Plasmid constructions. The plasmids used in this study are described in Table 2. pRM102 was constructed by amplifying yeast genomic DNA with primers 5'-ATGGATCCGCTGCACGGTCTGTTCCTA-3' and 5'-TTGTCGACAC CACCAGAACGGCCGTTAGA-3' and inserting the amplified sequence into pRS314 (38) at the *Bam*HI and *Sal*I restriction sites. This sequence includes the entire *HIS3* gene, as well as the first 236 bp of the *PET56* gene and the first 259 bp of the *DED1* gene. The *HIS3* promoter in this amplified sequence was modified such that it contained both an *Eco*RI site and a *Cla*I site inserted between the TATA box and the translation start site. This modification had no detectable effect on *HIS3* expression and is mentioned solely to indicate its presence. The *pet56* and *ded1* sequences are oriented such that both promoters directly flank and are directed away from the *HIS3* gene. pRM101 was made by inserting the same amplified *HIS3* sequence into the pGEM-11Zf (Promega) vector with the *Bam*HI and *Sal*I restriction sites. The resulting plasmid was then

TABLE 1. Yeast strains used in this study

Strain	Genotype ^a	Source
RMY057	<i>MATα</i> W303(pRS314)	This study
RMY063	<i>MATα hpr1Δ::HIS3</i> W303(pRS314)	This study
RMY065	<i>MATα</i> W303(pRM101)	This study
RMY067	<i>MATα hpr1Δ::HIS3</i> W303(pRM101)	This study
RMY064	<i>MATα</i> W303(pRM102)	This study
RMY066	<i>MATα hpr1Δ::HIS3</i> W303(pRM102)	This study
RMY083	<i>MATα</i> W303(pRM103)	This study
RMY085	<i>MATα hpr1Δ::HIS3</i> W303(pRM103)	This study
RMY079	<i>MATα</i> W303(pRM104)	This study
RMY081	<i>MATα hpr1Δ::HIS3</i> W303(pRM104)	This study
RMY078	<i>MATα hpr1Δ::HIS3</i> W303(pRM110)	This study
RMY076	<i>MATα hpr1Δ::HIS3</i> W303(pRM111)	This study
RMY206	<i>MATα</i> W303(pRM115)(YE- <i>SUB2</i>)	This study
RMY207	<i>MATα hpr1Δ::HIS3</i> W303(pRM115)(YE- <i>SUB2</i>)	This study

^a All strains are in the W303 genetic background (*leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can1-100 RAD5*).

digested with *Apa*I and *Sac*I. The resulting *pet56-HIS3-ded1* fragment was purified from the digest and then reinserted into pRS314 at the *Apa*I and *Sac*I restriction sites, resulting in an inversion of the inserted yeast sequence.

pRM103 is identical to pRM102, except that the *ded1* fragment, along with the promoter, has been excised from the plasmid with the *Bss*HII and *Sal*I restriction sites. It is important to note that this procedure does not remove the major *HIS3* mRNA termination site (42). The restriction sites were processed with Klenow fragment to generate blunt ends that were subsequently ligated together. pRM104 is identical to the pRM102 plasmid, except that the *HIS3-pet56* region has been removed from the plasmid with the *Bss*HII and *Bam*HI restriction sites. Again, the overhanging ends were processed with Klenow fragment and the plasmid was religated at these blunt ends. Therefore, pRM104 contains the *ded1* fragment together with the *DED1* promoter but lacks the *HIS3* and *pet56* sequences.

pRM110 is pRM102 with a *CYC1* transcriptional terminator (26) inserted directly after the *ded1* sequence at the *Sal*I and *Apa*I restriction sites. The *CYC1* terminator was amplified by PCR from the yeast genome with primers 5'-CGCGGGTCTCTGTCGACCGATATCATGTAATTAGTTATGTCACGC TTAC-3' and 5'-CGCAGGACCCGGGCCCCGCGACGATGAGAGTGTA ACTGCGAAGCTTGC-3', which contain *Sal*I and *Apa*I restriction sites, respectively, at the 5' end. The orientation of the terminator is identical to that of wild-type *CYC1* relative to the direction of transcription. Both of the primers also contain a *Psp*511 restriction site, which was used to construct pRM111 with the PCR-amplified *CYC1* transcriptional terminator. pRM111 contains the *CYC1* terminator inserted into the *Psp*511 restriction site of pRM102, almost directly between the *Escherichia coli amp* and yeast *CEN6* sequences. pRM112 has the *CYC1* terminator inserted between the *ori* and *amp* sequences of pRM102 at the *Nco*I restriction site.

pRM115 is pRM102 with 630 bp of the *E. coli dnaB* sequence inserted into the *Sal*I restriction site. The *dnaB* sequence was PCR amplified with the primers (each containing a *Sal*I site at the 5' end) 5'-CATGGTCGACCCCTTCAACA AACAGCAGGCTGAAC-3' and 5'-CATGGTCGACGTTGAGATCGTCATA ACCGGTGTTCAC-3' and inserted into the *Sal*I site of pRM115 such that the sequence was in the reverse orientation relative to the direction of transcription. This plasmid was constructed so that the *dnaB* sequence could be used to probe for plasmid *DED1* promoter transcription in the presence of high-copy *hpr1* suppressor *SUB2*.

Plasmid loss rates. Plasmid loss rates of strains containing pRM102 (*CEN TRP*) and its derivatives were calculated as described previously in detail (10). Briefly, cells were grown in liquid SC lacking tryptophan (SC-Trp) to mid-log phase. Equal aliquots were taken and plated onto both SC and SC-Trp plates to determine the percentage of plasmid-containing cells. The culture was then diluted 1:1,000 into liquid SC to release the plasmid selection and allowed to grow to stationary phase. Again, equal aliquots were plated onto SC and SC-Trp to determine the percentage of cells that still contained the plasmid. Plasmid loss rates (*m*) were calculated with the formula $m = 1 - e^{\ln(P_2/P_1)/g}$, where *P*₁ and *P*₂ are the percentages of plasmid carrying cells before and after the release of selection, respectively. *g* is the number of doublings during nonselective growth and is described as $g = \ln(N_2/N_1)/\ln 2$. *N*₁ equals the number of viable cells per

TABLE 2. Plasmids used in this study

Name	Description ^a	Source
pRS314	<i>CEN6 ARSH TRP1 E. coli ori-amp</i>	R. Sikorski
pRM101	1.7-kb <i>ded1-HIS3-pet56</i> fragment in pRS314 with <i>PET56</i> promoter oriented toward <i>ori-amp</i>	This study
pRM102	1.7-kb <i>ded1-HIS3-pet56</i> fragment in pRS314 with <i>DED1</i> promoter oriented toward <i>ori-amp</i>	This study
pRM103	pRM102 with <i>ded1</i> and its promoter removed	This study
pRM104	pRM102 with <i>HIS3-pet56</i> removed	This study
pRM110	pRM102 with <i>CYC1</i> transcription terminator inserted directly after <i>ded1</i>	This study
pRM111	pRM102 with <i>CYC1</i> transcription terminator inserted directly in front of <i>GEN6</i>	This study
pRM112	pRM102 with <i>CYC1</i> transcription terminator inserted between <i>ori</i> and <i>amp</i>	This study
pRM115	pRM102 with 630 bp of <i>E. coli dnaB</i> sequence, inserted 3' to <i>ded1</i>	This study
YEp-SUB2	<i>SUB2</i> cloned into YEp351	H. Fan

^a See Materials and Methods for details of each plasmid.

milliliter before nonselective growth, and N_2 equals the number of viable cells per milliliter after nonselective growth (viable cells are cells growing on SC). The significance of differences between the plasmid loss rates was analyzed with the Student *t* test.

Transcriptional run-on analysis. Transcriptional run-on analysis was performed as previously described (46), with some modifications (7). Cultures were grown under plasmid selection in liquid medium to mid-log phase and separated into two aliquots, one for DNA analysis and one for transcriptional run-on analysis. For the run-on analysis, 3×10^7 cells were chilled by being poured over sterile ice and collected by centrifugation at $2,860 \times g$ and 4°C . Cells were then washed in 5 ml of cold (0°C) TMN buffer (10 mM Tris [pH 7.4], 100 mM NaCl, 5 mM MgCl_2) and collected by centrifugation. The cells were resuspended in 0.95 ml of 0°C sterile H_2O and transferred to a microcentrifuge tube. A 0.05-ml volume of 10% *N*-lauroyl sarcosine was added, and the tube was left on ice for 15 min. The tube was gently centrifuged at 4°C ($1,830 \times g$ for 1 min), and the supernatant was removed. The cells were then resuspended in 0.15 ml of a reaction mixture containing 20 mM Tris (pH 7.7); 200 mM KCl; 32 mM MgCl_2 ; 2 mM dithiothreitol; 0.5 mM (each) ATP, CTP, and GTP; and 120 μCi of [^{32}P]UTP ($>3,000$ Ci/mmol) and incubated for 7.5 min at room temperature. The reaction was stopped by addition of 1 ml of cold (4°C) TMN buffer containing 1 mM unlabeled UTP. Cells were collected, and RNA was isolated with hot acidic phenol (20).

Probes (5 μg of each) for the run-on analysis were immobilized to positively charged Hybond-XL nylon membranes (Amersham Pharmacia Biotech) as previously described (11). Each probe was generated via PCR amplification and gel purified. The primers used to generate probes for pRM102 were 5'-CTGTCGTGCCAGTGCATTAATG-3' and 5'-CCGAAGGGAGAAAGGCGGACAGG-3'; 5'-AGCGTGGCGCTTTCTCAATGCTC-3' and 5'-CTCATGACCAAAAATCCCTTAACGTGAG-3'; 5'-TTATCAAAAAGGATCTTCACCTAG-3' and 5'-ATACACTATTCTCAGAATGACTTGG-3'; 5'-GGCGACCGAGTTGCTCTTGC-3' and 5'-GTAAAATCACAGGATTTTCGTG-3'; and 5'-TTACTTATCGTAAATCGAATG-3' and 5'-ATGGTGCACCTCAGTACAATC-3'.

The rRNA gene control probe (*RDN25*) used in these experiments was also generated via PCR amplification with primers 5'-CAACCGGATTGCCTTAATAAC and TTACGCGTATGGGTTTACACC-3'. The primers used to generate the probes for pRM119 were 5'-CAGCTTTTGTCCCTTTAGTG-3' and 5'-CCGAAGGGAGAAAGGCGGACAGG-3'; 5'-AGCGTGGCGCTTTCTCAATGCTC-3' and 5'-ATACACTATTCTCAGAATGACTTGG-3'; and 5'-GGCGACCGAGTTGCTCTTGC-3' and 5'-ATGGTGCACCTCAGTACAATC-3'. The *ACT1* control probe was generated with the primers 5'-ACTAACATCGATTGCTTCATTC-3' and 5'-TTCTCAAAATGGCGTGAGGTAG-3'.

Isolated RNAs containing [^{32}P]UTP from the run-on experiments were hybridized to the filters with the immobilized probes in $5\times$ SSC (0.75 M NaCl, 0.075 M Na citrate)– $5\times$ Denhardt's solution (0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin fraction V)–50% formamide–1% sodium dodecyl sulfate (SDS)–100 ng of single-stranded salmon sperm DNA at 42°C overnight.

Northern and Southern analyses. RNA was isolated from exponential-phase cultures growing in the appropriate selective medium with hot acidic phenol (20). Northern analysis was performed with formaldehyde agarose gels by standard procedures (33). RNAs were transferred to and immobilized on Hybond-XL nylon membranes (Amersham Pharmacia Biotech). The appropriate probe was hybridized to the transferred RNAs in $5\times$ SSC– $5\times$ Denhardt's solution–50% formamide–1% SDS–100 μg of single-stranded salmon sperm DNA per ml at 42°C overnight.

Genomic and plasmid DNAs were isolated from yeast in the logarithmic growth phase as previously described (37). Southern analysis was performed in accordance with standard procedures (33). Gels were transferred to and immobilized on Hybond-XL nylon membranes. The appropriate probes were hybridized to the transferred material in $5\times$ SSC– $5\times$ Denhardt's solution–1% SDS–100 μg of single-stranded salmon sperm DNA per ml at 68°C overnight.

The *DED1* promoter plasmid transcript was detected with a probe synthesized from a fragment (generated by a PCR with primers 5'-CTGTCGTGCCAGTGCATTAATG-3' and 5'-CCGAAGGGAGAAAGGCGGACAGG-3') of the plasmid bacterial *ori* sequence. This same probe was also used to detect plasmid DNA by Southern analysis. To detect the *DED1* promoter plasmid transcript with pRM115, a probe was synthesized from the *SalI* *dnaB* fragment of pRM115. Detection of actin mRNA on Northern blots, as well as the *ACT1* DNA sequences on Southern blots, was done with the same *ACT1* sequence as that used for the run-on analysis. All probes were generated by random priming and labeled with [α - ^{32}P]dCTP. All blots, including those from the run-on analysis, were visualized with a Bio-Rad Molecular Imager FX and quantitated with NIH Image 1.61.

Quantitative RT-PCR methods. Reverse transcription (RT)-PCRs were performed such that the transcript produced from the *DED1* promoter present on pRM102 could be detected and quantitated from the five different RT primers shown in Fig. 5. As a control, each of the RT reaction mixtures contained the same amount of RNA isolated from *E. coli* overexpressing *dnaB*. This was done so that the variation between each RT and the subsequent PCR could be measured and proper quantitation of the *DED1* promoter transcript from pRM102 could be achieved. Overexpression of *dnaB* in *E. coli* strain BL21(DE3) was achieved with expression plasmid pET3C containing a copy of *dnaB* (43).

All yeast cultures were grown to mid-log phase in liquid SC-Trp. Each culture was split in half so that RNA could be isolated from one half and DNA could be isolated from the other. RNA was isolated with hot acidic phenol (20). DNA was isolated by a standard protocol (37) and used to determine the relative amount of plasmid present in each strain. RT-PCR was performed with the ThermoScript RT-PCR System (Life Technologies). The RT reaction mixture contained 1 μg of total yeast RNA; 0.1 μg of total *E. coli* RNA; 0.2 μM 3' primer; 1 $\times$ ThermoScript cDNA synthesis buffer; 1 $\times$ ThermoScript RNaseOUT; 250 μM each dATP, dCTP, dGTP, and dTTP; and 15 U of ThermoScript reverse transcriptase. The RNA and 3' primer were first incubated at 65°C in 10 μl of diethyl pyrocarbonate-treated water for 5 min to denature the RNA and the primer. The rest of the components were added to create a 20- μl reaction mixture, which was then incubated at 50°C for 60 min. The reaction was terminated by incubation at 85°C for 5 min. Two units of RNase H was added, and the mixture was incubated at 37°C for 20 min. Two microliters of this RT reaction mixture was subsequently used for the PCR along with 0.2 μM each primer; 100 μM each dATP, dCTP, dGTP, and dTTP; 1 $\times$ manufacturer's PCR buffer; and 2.5 U of Expand Long Template polymerase mixture (Boehringer Mannheim). Normal cycling times were 93°C for 30 s, 55°C for 30 s, and 72°C for 1 min 30 s. Most reactions were cycled 35 times. Five different 3' RT primers were used and designed such that they would hybridize to the transcript produced from the *DED1* promoter present on pRM102. These primers were as follows: RT primer 1, 5'-GTTTCCCGACTGGAAAGCGGGCAGTG-3'; primer 2, 5'-CTCATGACCAAAATCCCTTAACGTGAG-3'; primer 3, 5'-ATAGACTGGATGGAGGCGGATAAA G-3'; primer 4, 5'-ATACACTATTCTCAGAATGACTTGG-3'; primer 5, 5'-TGTATCCGCTCATGAGACAATAACCC-3'. The *dnaB* RT control primer, 5'-GCAATTTCCAGCGTACGGTTATCGG-3', was also added to each tube.

The subsequent five PCRs were performed with one each of the above 3'

primers and a single 5' primer (5'-CATATTATGGCTGAAGTGAAC-3') that hybridizes to the *DED1* fragment present on pRM102, 110 bp downstream from the translation start site. In addition, to each of the five PCRs, specific control *dnaB* primers 5'-CCCTTCAACAACAGCAGGCTGAAC-3' and 5'-GCAATTTCCAGCGTACGGTTATCGG-3' were also added.

Exactly the same amount of each PCR mixture was run on 1% agarose gels and subjected to Southern analysis. Each RT-PCR was visualized with a radiolabeled probe made from gel-purified PCR amplifications of the plasmid backbone sequence on pRM102 with primers 5'-GGCCCGGTACCCAGCTTTT G-3' and 5'-GTTTCCCGACTGGAAAGCGGGCAGTG-3' (Fig. 5). *dnaB* was visualized with a radiolabeled probe made from gel-purified PCR amplifications of *dnaB* with primers 5'-CCCTTCAACAACAGCAGGCTGAAC-3' and 5'-GCAATTTCCAGCGTACGGTTATCGG-3'.

Northern analysis probing for actin mRNA was performed on the material initially isolated to determine the relative levels of total RNA. Radiolabeled probe was synthesized from purified *ACT1* PCR amplifications with primers 5'-ACTAACATCGATTGCTTCATTC-3' and 5'-TTCTCAAATGGCGTGA GGTAG-3'.

The relative amount of plasmid present in each strain was determined by Southern analysis. Approximately 1.0 μ g of DNA from each isolation was digested with *EcoRI* for 4 h at 37°C and resolved on a 1% agarose gel. *EcoRI* cuts the plasmid once and cuts chromosomal DNA such that a 4.0-kb fragment containing *ACT1* is produced. DNA blots were simultaneously hybridized with radiolabeled probes designed to recognize plasmid pRM102, as well as a single-copy chromosomal sequence, *ACT1*. pRM102 was visualized with the same probe used to detect the above-described RT-PCR products. *ACT1* DNA was visualized with the same probe used as described above to detect actin mRNA.

Relative levels of the RT-PCR amplification products were quantitated and normalized against relative actin mRNA levels, the relative amount of plasmid present, and the relative efficiency of the *dnaB* RT-PCR. All hybridizations were visualized with a Bio-Rad Molecular Imager FX and quantitated with NIH Image 1.61 software.

DNA enzymatic manipulations. All restriction digests were performed in accordance with the manufacturer's recommendations. All PCR amplifications were performed with the Expand Long Template PCR System and standard protocols (Roche Diagnostics). All PCR products were gel purified with 1% agarose gels and the QIAquick Gel Extraction Kit from Qiagen.

RESULTS

Transcription affects plasmid stability in *hpr1*. Two plasmids were constructed by inserting a 1.7-kb yeast chromosomal sequence into the multiple cloning site of single-copy yeast plasmid pRS314. The two plasmids differ only in the orientation of the insert, which contains the entire *HIS3* gene, along with approximately 250 bp of the 5' ends of both *DED1* and *PET56*. *DED1* and *PET56* directly flank *HIS3* and are oriented such that both promoters are directed away from *HIS3* (Fig. 1). These two plasmids differed markedly in stability when carried by an *hpr1* Δ strain, while both were stable in the wild-type strain (Fig. 1). To quantitate these observations, the rate of loss of each plasmid in each strain was determined (Table 3). We have previously reported plasmid loss rates for pRM102 in both the wild-type and *hpr1* Δ strains (15). We repeated these experiments and expanded upon these observations.

pRM101 has the same stability when it is present in both wild-type and *hpr1* Δ cells, and the loss rate in each of these strains is not significantly different from that of the empty vector (pRS314) alone (Table 3). However, pRM102 is more than eight times decreased in stability when present in *hpr1* Δ cells than when present in wild-type cells ($P < 0.001$). To determine the cause of the instability of pRM102 in an *hpr1* Δ mutant strain, we deleted various regions of the 1.7-kb insert. Deletion of the *PET56* fragment along with its promoter had no effect on the instability of pRM102 in an *hpr1* Δ strain (data not shown). Furthermore, removal of *HIS3* with the *PET56*

FIG. 1. A yeast chromosomal insert confers differential plasmid stability in an *hpr1* Δ background, dependent on its orientation in the plasmid. pRM101 and pRM102 differ in the orientation of a 1.7-kb yeast chromosomal insert. The *PET56* and *DED1* genes are both oriented away from *HIS3* and are designated *pet56* and *ded1* solely to indicate that they are truncations. The photograph above each plasmid diagram gives a qualitative representation of its respective stability in *hpr1* Δ cells. *hpr1* Δ cells containing the plasmid were taken from selective (SC-Trp) plates, streaked onto nonselective rich medium (YPD) plates, and then replica plated back onto selective (SC-Trp) plates. An example of a colony that has lost the plasmid is indicated by the white arrow.

fragment from pRM102 (to create pRM104) also did not rescue the instability of this plasmid in an *hpr1* Δ mutant strain. In fact, pRM104 has an average loss rate of 29.1×10^{-2} and is much more unstable in *hpr1* Δ cells than is pRM102 ($P < 0.001$) (Table 3). Since pRM104 has only the *DED1* fragment and its promoter inserted in the plasmid, this suggests that the main cause of the instability of pRM102 in *hpr1* Δ mutant cells is the *DED1* promoter. To confirm this, we deleted the *DED1* fragment, along with its promoter, from the pRM102 plasmid to create pRM103. This cured the instability of this plasmid in *hpr1* Δ cells (Table 3).

To determine why the *DED1* promoter oriented toward the bacterial *ori-amp* sequence in pRM102 causes this plasmid to be unstable in *hpr1* Δ cells, we examined the transcripts from the *DED1* promoter in pRM102 and transcripts from the *PET56* promoter in pRM101 in both wild-type and *hpr1* Δ cells (Fig. 2). These Northern assays indicate that the *DED1* promoter is capable of producing an approximately 2.5-kb transcript when it is oriented toward the *ori-amp* sequence (pRM102) in both wild-type and *hpr1* Δ cells. However, when the *PET56* promoter is oriented toward the *ori-amp* sequence, this large transcript is not produced. A 2.5-kb transcript corresponds to a transcript starting from the *DED1* promoter and terminating in front of the *CEN6 ARSH4* region of the plasmid. This was confirmed by Northern analysis with probes

TABLE 3. Plasmid loss rates per cell per generation^a

Genotype	Avg plasmid loss rate, 10 ⁻² (SD)
<i>HPR1</i> (pRS314).....	1.7 (1.4)
<i>hpr1Δ</i> (pRS314).....	3.3 (0.5)
<i>HPR1</i> (pRM101).....	2.3 (1.1)
<i>hpr1Δ</i> (pRM101).....	2.2 (1.2)
<i>HPR1</i> (pRM102).....	1.9 (0.4)
<i>hpr1Δ</i> (pRM102).....	16.2 (0.8)
<i>HPR1</i> (pRM103) (pRM102 minus <i>ded1</i>).....	1.6 (1.9)
<i>hpr1Δ</i> (pRM103) (pRM102 minus <i>ded1</i>).....	2.7 (1.3)
<i>HPR1</i> (pRM104) (pRM102 minus <i>HIS3-pet56</i>).....	3.8 (1.3)
<i>hpr1Δ</i> (pRM104) (pRM102 minus <i>HIS3-pet56</i>).....	29.1 (2.1)
<i>hpr1Δ</i> (pRM110) (pRM102 with <i>CYC1</i> terminator after <i>ded1</i>).....	1.4 (0.8)
<i>hpr1Δ</i> (pRM111) (pRM102 with <i>CYC1</i> terminator before <i>CEN6</i>).....	8.1 (0.7)
<i>hpr1Δ</i> (pRM112) (pRM102 with <i>CYC1</i> terminator between <i>ori</i> and <i>amp</i>).....	1.8 (0.5)

^a Plasmid loss rates of strains tested in this study are shown. The values averages of three independent experiments., pRM102 is a pRS314 vector with a 1.7-kb chromosomal insert that flanks the yeast *HIS3* gene. Plasmid pRM103 is missing the *ded1* sequence and promoter. Plasmid pRM104 is missing the *HIS3-pet56* sequence, leaving only the *ded1* sequence and promoter. pRM110 terminates *DED1* promoter transcription prior to entering *ori-amp* sequences. pRM111 terminates transcription after elongation through the *ori-amp* sequence. pRM112 terminates transcription after *ded1* and before *CEN6*, between the *ori* and *amp* sequences.

flanking the *CEN6 ARSH4* sequence. Only a probe in front of *CEN6* would hybridize to the transcript (data not shown). Therefore, the *DED1* promoter present on pRM102 is capable of producing a transcript that can progress at least through the bacterial *ori-amp* sequence on the plasmid. These experiments suggest that the *DED1* promoter is relatively stronger than the *PET56* promoter, consistent with previously published results (44).

The data in Fig. 2 suggest that transcription elongation through the *ori-amp* sequence may be the cause of the plasmid

instability in *hpr1Δ* cells. To test this hypothesis, we inserted a strong *CYC1* transcriptional terminator at three different sites of pRM102. First, we inserted the terminator directly 3' to the *DED1* fragment on pRM102 to create pRM110 (Fig. 3). Qual-

FIG. 2. Analysis of transcription through the bacterial *ori-amp* sequence on both pRM102 and pRM101. Northern analysis was performed to identify transcripts originating from the *DED1* promoter (pRM102, lanes 1 through 6) and the *PET56* promoter (pRM101, lanes 7 through 12) with a DNA probe from the bacterial *ori* sequence on the plasmid. RNA was isolated from three independent cultures grown to mid-log phase in selective (SC-Trp) medium for both wild-type (wt) and *hpr1Δ* strains each containing either pRM102 or pRM101. The arrow points to the major transcript (approximately 2.5 kb) that is generated from the *DED1* promoter on pRM102 (lanes 1 to 6). This major transcript is not observed when the *PET56* promoter is oriented toward the bacterial *ori-amp* sequence (pRM101, lanes 7 to 12). Relative actin mRNA levels from each culture are shown at the bottom

FIG. 3. Early termination of transcription from the *DED1* promoter suppresses plasmid instability in *hpr1Δ* cells. *CYC1* transcription terminators were inserted either directly after the *ded1* sequence on pRM102 (pRM110), directly in front of the *CEN6* sequence (pRM111), or between the *ori* and *amp* sequences (pRM112) (see Materials and Methods). Given are qualitative assessments of the relative stability of each plasmid in an *hpr1Δ* background.

itative observations indicated that this completely stabilized the plasmid in *hpr1Δ* cells. Quantitation of the rate of plasmid pRM110 loss confirmed this and showed that this transcriptional terminator completely suppresses the instability of pRM102 in *hpr1Δ* cells (Table 3). Northern analysis confirmed that the *CYC1* terminator efficiently blocked all detectable transcription initiating from the *DED1* promoter on pRM110 (data not shown). We next inserted the *CYC1* transcriptional terminator directly in front of the *CEN6* sequence on pRM102 to create pRM111 (Fig. 3). pRM111 remained relatively unstable in *hpr1Δ* cells compared to pRM110 (Table 3) ($P < 0.001$). Northern analysis of the transcript generated from pRM111 confirmed that transcription progressed through the *ori-amp* sequence and terminated at the *CYC1* terminator sequence. Finally, the *CYC1* transcription terminator was inserted between the *ori* and *amp* sequences to create pRM112 (Fig. 3). Plasmid instability was suppressed in *hpr1Δ* cells (Table 3) ($P < 0.001$). Therefore, termination of transcription from the *DED1* promoter prior to progression through the bacterial *ori-amp* sequence on pRM102 suppresses the instability in an *hpr1Δ* strain. However, allowing transcription elongation to pass through the *ori-amp* sequence and terminate downstream maintains the instability of pRM102 in *hpr1Δ* cells. The finding that the pRM112 plasmid with a transcription terminator between the *ori* and *amp* sequences is stable in *hpr1Δ* cells rules out aberrant chromatin changes during the initiation of transcription as the cause of plasmid instability.

Plasmid transcript levels are not reduced in *hpr1Δ* cells. Previous studies with plasmid-based systems have determined that Hpr1p-deficient cells have difficulty with transcription elongation through a variety of DNA sequences and often result in a transcription block that is correlated with genetic instability between direct repeats (7, 32). If transcription from the *DED1* promoter on pRM102 in *hpr1Δ* cells were somehow blocked within the *ori-amp* sequence, then we would expect to see a reduction in steady-state levels of the full-length transcript. The Northern analysis in Fig. 2 suggests that although the 2.5-kb transcript can be detected in *hpr1Δ* cells carrying pRM102, the levels are reduced compared to those in the wild type. However, proper quantitation of this transcript by correction against the amount of plasmid present, as well as the level of actin mRNA and actin DNA, reveals that the levels of the *DED1* promoter transcript are not reduced in *hpr1Δ* cells compared to those in wild-type cells (Fig. 4). Figure 4 shows the results of three independent experiments. The absolute levels of the *DED1* promoter transcript are not reduced in *hpr1Δ* cells relative to those in wild-type cells because the actual levels of pRM102 are very low in *hpr1Δ* cells, even under selection, because of its inherent instability. On the contrary, these results demonstrate that there might be a slight increase in the absolute levels of this transcript in *hpr1Δ* cells relative to those in wild-type cells. Although these increases are small, we suspect that they are not trivial. We have observed this slight increase in numerous other Northern experiments, as well as in quantitative RT-PCR experiments designed to detect levels of the *DED1* promoter transcript from pRM102.

Quantitative RT-PCR was performed to show that the transcripts observed in Fig. 4 were derived from the *DED1* promoter on pRM102. These experiments also confirmed the quantitation obtained by Northern analysis. The locations of

FIG. 4. Quantitation of the major transcript produced from the *DED1* promoter on pRM102 in both wild-type (wt) and *hpr1Δ* strains. (A) Northern analysis of transcription from the *DED1* promoter present on pRM102 in both wild-type and *hpr1Δ* strains. RNA was isolated from three independent cultures of both wild-type and *hpr1Δ* strains, each containing pRM102, grown to mid-log phase in liquid SC-Trp. The transfers were probed with the same bacterial *ori* probe as in Fig. 2. The arrow points to the major transcript (approximately 2.5 kb) that is generated from the *DED1* promoter on pRM102. (B) Southern analysis to determine the relative amount of plasmid present in each strain. DNA was isolated from each of the same cultures used for the above Northern assay. The DNAs were digested with *Bam*HI, and equal amounts were loaded onto a 0.9% agarose gel. The transferred materials were simultaneously probed for plasmid (with the bacterial *ori* probe), as well as a single-copy yeast gene (*ACT1*). (C) Quantitation of the *DED1* promoter transcript levels present in *hpr1Δ* cells relative to those in the wild type. Relative levels of transcript produced from the *DED1* promoter were normalized against relative actin mRNA levels, as well as the relative amount of plasmid present in each strain. The relative amount of plasmid was determined by comparing the plasmid levels against that of a single-copy chromosomal sequence (*ACT1*).

five RT primers used in this experiment are shown in Fig. 5. Each primer was used with the 5' PCR primer to generate five different RT-PCR products, which are shown in Fig. 5B. Quantitation of the results of three separate experiments showed that the steady-state level of the pRM102 *DED1* promoter transcript in the *hpr1Δ* strain does not differ from that observed in the wild-type strain (Fig. 5C).

Therefore, although we determined that transcription elon-

FIG. 5. Quantitative RT-PCR analysis of transcription from the *DED1* promoter of pRM102. (A) Schematic of the locations of the five RT primers used to analyze the *DED1* promoter transcript. (B) Southern analysis of the RT-PCR products of the five reactions from wild-type and *hpr1Δ* cells carrying pRM102. The position of the *dnaB* transcript used as a PCR efficiency control is indicated. Relative levels of plasmid and total mRNAs present in each reaction were determined by Southern and Northern analyses, respectively. (C). Quantitated RT-PCR levels of *hpr1Δ*(pRM102) cells relative to those of wild-type cells carrying pRM102 are shown. The results are from three independent experiments for each reaction. The error bars indicate standard deviations. Absolute RT-PCR levels were corrected for the amount of plasmid present in each strain and for the total amount of mRNA. chrom., chromosome.

gation from the *DED1* promoter on pRM102 is required to observe the plasmid instability in an *hpr1Δ* strain, we could not detect any evidence of a transcriptional defect by Northern analysis or RT-PCR.

Plasmid instability in *hpr1Δ* cells is increased in the presence of 6-AU. To further demonstrate that the instability of pRM102 in *hpr1Δ* cells is directly related to transcription, we tested the stability of the plasmid in the presence of 6-AU. 6-AU decreases intracellular pools of GTP and UTP by inhibiting components of their biosynthetic pathways (12, 17). Consequently, 6-AU sensitivity is often correlated with mutations in components of the RNA polymerase II transcriptional machinery involved in elongation (17). In the presence of 6-AU, pRM102 was extremely unstable in *hpr1Δ* cells. The slow growth of this strain made the calculation of a plasmid loss rate impractical. To demonstrate instability, we calculated the fraction of cells possessing plasmid both in the presence and in the

TABLE 4. Plasmid stability in cultures containing 6AU

Genotype	Avg proportion of cells with plasmid (SD) ^a	
	With 6-AU	Without 6-AU
<i>HPR1</i> (pRM101)	0.91 (0.07)	0.91 (0.03)
<i>hpr1Δ</i> (pRM101)	0.78 (0.02)	0.88 (0.05)
<i>HPR1</i> (pRM102)	0.81 (0.08)	0.62 (0.06)
<i>hpr1Δ</i> (pRM102)	0.05 (0.01)	0.38 (0.02)

^a Proportions of wild-type and *hpr1Δ* strains carrying plasmids pRM101 and pRM102 in the presence and absence of 6-AU are shown. All strains are *URA3⁺* and were grown in liquid SC-Trp-Ura with or without 100 μg of 6-AU per ml to mid-log phase. Cells were then plated on SC plates and allowed to grow for 3 days at 30°C. The plates were then replica plated to SC-Trp plates to determine the proportion of colonies still containing pRM102. The values shown are averages of at least three independent experiments.

absence of 6-AU while the cells were grown in liquid medium selecting for the plasmid. pRM101 is relatively stable in *hpr1Δ* cells and is not affected by the addition of 6-AU (Table 4). Consequently, pRM102 is much more unstable in *hpr1Δ* cells when 6-AU is present in the growth medium.

High-copy-number *SUB2* suppresses plasmid instability in *hpr1Δ* cells and increases steady-state plasmid *DED1* promoter transcript levels relative to those in the wild type. DEAD box-containing putative RNA helicase-encoding gene *SUB2*, when present on a high-copy-number plasmid, suppresses *hpr1Δ*-stimulated recombination events between direct repeats and the increased plasmid loss rate of pRM102 (15). This suppression is extremely strong and results in plasmid loss rates that are close to the wild-type levels. *SUB2* suppression is easily visualized with Southern analysis by comparing the relative plasmid levels in *hpr1Δ* mutant and wild-type cells (Fig. 6). Since high-copy-number *SUB2* has such a strong stabilizing effect on pRM102 in *hpr1Δ* cells, we wanted to determine its effect on the steady-state levels of the plasmid *DED1* promoter transcript. For this, we inserted a short unique DNA sequence directly 3' to the *ded1* sequence on pRM102 (to create pRM115, which is identical to pRM102 with regard to plasmid instability in *hpr1Δ* cells) to use as a probe for the *DED1* promoter transcript. We used the unique sequence as a probe since the plasmid containing *SUB2* (YE-p-SUB2) contains the same bacterial *ori* sequence used to probe for the pRM102 *DED1* promoter transcript. Surprisingly, in the presence of high-copy-number *SUB2*, *hpr1Δ* cells displayed significantly increased steady-state levels of the *DED1* promoter transcript from pRM115 compared to those in wild-type cells ($P < 0.01$) (Fig. 6). This suggests that the *DED1* promoter on pRM115 may actually be more accessible to the RNA polymerase II transcriptional machinery in *hpr1Δ* cells. We do not know how high-copy-number *SUB2* stimulates transcription in an *hpr1Δ* strain. Presumably, the putative RNA helicase activity is involved in this process. We have previously shown that Sub2p associates with RNA polymerase II when it is overexpressed in *hpr1Δ* cells (15).

Transcriptional run-on analysis of plasmid transcripts in *hpr1Δ* cells. To further elucidate the relationship between transcriptional elongation through the *ori-amp* sequence in pRM102 and its instability in *hpr1Δ* cells, we performed a transcriptional run-on analysis with probes covering the entire

FIG. 6. High-copy-number *SUB2* suppresses plasmid instability and increases the relative amount of the major transcript produced from the *DED1* promoter on pRM115 in *hpr1Δ* cells. pRM115 is identical to pRM102 except that 630 bp of the *E. coli dnaB* sequence is inserted directly 3' to the *ded1* sequence so that the plasmid *DED1* promoter transcript could be uniquely probed in the presence of the high-copy-number YEp-*SUB2* plasmid (YE351 backbone). (A) Northern analysis of transcription from the *DED1* promoter present on pRM115 in both wild-type (wt) and *hpr1Δ* strains in the presence of high-copy-number YEp-*SUB2*. RNA was isolated from three independent cultures grown to mid-log phase in liquid SC-Trp-Leu. The same amount of RNA was loaded onto each lane, and transfers were probed with the bacterial *dnaB* probe. The blots were subsequently stripped and reprobed for actin mRNA. (B) Southern analysis to determine the relative amount of plasmid pRM115 present in each strain. DNA was isolated from each of the same cultures used for the above Northern assay. DNAs were digested with *EcoRI*. The transfer was simultaneously probed for pRM115 (*dnaB* probe) and a single-copy yeast gene (*ACT1*). (C) Quantitation of the *DED1* promoter transcript levels present in *hpr1Δ* cells relative to those in wild-type cells. Relative levels of transcript produced from the *DED1* promoter were normalized against relative actin mRNA levels, as well as the relative amount of plasmid present in each strain.

ori-amp sequence, as well as the downstream *CEN6-ARSH4* sequence (Fig. 7C). The results of these experiments are shown in Fig. 7A. A representative blot is shown on the left, while the averages and standard deviations of four independent experiments are shown on the right. These results suggest that an increased RNA polymerase II density is present throughout the length of these sequences in *hpr1Δ* cells compared to that in wild-type cells. The relative levels on each blot were corrected for both the amount of plasmid present in the cells and

FIG. 7. Transcriptional run-on analysis of the plasmid *DED1* promoter transcript in *hpr1Δ* and wild-type cells. Cells were grown under selection in liquid medium (SC-Trp) to mid-log phase prior to run-on analysis. (A) Radiolabeled RNA from both wild-type (wt) and *hpr1Δ* cells, each containing pRM102, bound to each probe. Transcription of rRNA was used to normalize transcription levels. The arrow indicates the direction of transcription. The location of each probe from the plasmid is shown in panel C. The bar graph shows the average level of transcription across each probe from four independent experiments for *hpr1Δ* cells relative to wild-type cells, normalized against both the total amount of RNA (25S rRNA) and the relative amount of plasmid present in each strain (B). Error bars represent the standard deviations. (B) Southern analysis to determine the relative amount of plasmid pRM102 present in each strain. DNA was isolated from each of the same cultures used for the above run-on analysis. DNAs were digested with *EcoRI*. The transfer was simultaneously probed for pRM102 (bacterial *ori* probe), as well as a single-copy yeast gene (*ACT1*). chrom., chromosome.

the total amount of RNA (25S rRNA). Transcription across *ACT1* was also used to normalize for the total amount of RNA in other run-on experiments with similar results (data not shown).

These results are surprising because previous transcriptional run-on experiments have shown a loss of RNA polymerase II density of transcription of the bacterial *lacZ* sequences in *Hpr1p*-deficient cells (7). These same data also provided evidence of a nonspecific transcription block within this sequence, suggesting that RNA polymerase II transcription may be more susceptible to transient pauses in *hpr1Δ*. In our experiments, however, we saw an increase in RNA polymerase II density, as well as no evidence of a transcription block when a *DED1* promoter was used to drive transcription across the *ori-amp* sequence.

Plasmid copy number in *hpr1Δ* cells. One possible explanation for the plasmid instability in *hpr1Δ* cells is that unchecked transcription might continue through the *CEN6* region and disrupt its centromere activity. Our Northern analyses did not show evidence of transcription in this region. However, disruption of the centromere function on pRM102 should make it segregate as a yeast replicating plasmid without a centromere,

a YRp plasmid, which is known to be unstable because of missegregation (28, 39). In this model, pRM102 plasmids would accumulate in mother cells, thereby increasing the plasmid copy number in cells containing plasmid pRM102. On the basis of our Southern analyses, we calculated that, under selective conditions, the copy number of plasmid pRM102 in *hpr1* Δ cultures is 0.26 ± 0.04 of the copy number found in wild-type cultures. From the plasmid loss rate determinations, we found that the proportion of cells in an *hpr1* Δ culture that carry plasmid pRM102 is 0.28 ± 0.08 . This indicates that there is approximately one copy of plasmid pRM102 in *hpr1* Δ cells that have plasmid DNA. This is inconsistent with a missegregation model in which cells that contain the plasmid have a greatly increased copy number, up to 50 plasmids per cell.

We also tested a sequestration model of *hpr1* Δ -mediated plasmid loss. Under this model, a frozen or blocked RNA polymerase II-plasmid DNA-nascent mRNA ternary complex might be complexed with the nuclear membrane such that the sequestered plasmid would not segregate correctly to the daughter cell. The trapped complex might make it difficult to recover plasmid DNA by standard isolation methods, such that the results of Southern analyses done to estimate plasmid copy number are underestimates. To test this, we isolated and quantitated the plasmid copy number by a method that can extract DNA from membrane fractions (16). This method did not increase the pRM102 plasmid yield in *hpr1* Δ cells.

Role of homologous recombination in plasmid loss. Another explanation for the plasmid loss in *hpr1* Δ cells is that aberrant transcription generates a damaged DNA substrate, in this case, plasmid pRM102, which is a target for homologous recombination. Recombination between sister chromatids of the plasmid would form a dicentric plasmid, which is known to be unstable because of mitotic nondisjunction events (21, 25). If this were the case, then elimination of recombination through a *rad52* Δ mutation should increase plasmid stability. We tested plasmid stability in *hpr1* Δ *rad52* Δ and *hpr1* Δ *rad50* Δ strains and found that plasmid stability was unaltered from that observed in an *hpr1* Δ strain, indicating that increased sister chromatid recombination is not the explanation for *hpr1* Δ -mediated plasmid instability.

DISCUSSION

These studies have shown that *hpr1* Δ cells have defects in the transcription of certain DNA sequences that can be related to problems in transcription elongation. These conclusions are similar to those of previous studies that found that *hpr1* Δ cells have difficulties in transcribing through portions of the bacterial *lacZ* sequence and parts of the plasmid *ori-amp* sequence (7, 32). The authors of those studies found evidence of transcriptional stalling, particularly when transcription was driven from the strong *GAL1* promoter under inducing conditions (7). We have observed that transcription through the plasmid *ori-amp* sequence results in instability of the plasmid when transcription is driven by the relatively strong constitutive *DED1* promoter. Deletion of the *DED1* promoter or insertion of a transcription terminator immediately 3' to the promoter restored plasmid stability.

However, in spite of the plasmid instability resulting from transcription elongation problems, we did not observe a de-

crease in message accumulation in *hpr1* Δ cells by Northern analysis. On the contrary, we observe equal, if not slightly higher, levels of the destabilizing transcript from the pRM102 *DED1* promoter in *hpr1* Δ cells, while the nuclear run-on experiment with the *DED1* promoter showed a relative increase in RNA polymerase II density in *hpr1* Δ cells. A possible explanation for this observation may involve our previous finding that polyadenylated RNA export from the nucleus to the cytoplasm is defective in *hpr1* Δ cells (34, 41). Northern analysis that measures mRNA accumulation would not reflect the RNA polymerase II transcripts that remain trapped in the nucleus and are a target for degradation (5, 48, 49). The increase in RNA polymerase II density in the *hpr1* Δ mutant could arise from increased accessibility of the *DED1* promoter to RNA polymerase II assembly in that promoter region. Increased accessibility in the *hpr1* Δ mutant could result from negative supercoiling at the promoter region behind the multiple RNA polymerase II complexes assembled on a circular DNA template at a strong promoter. We propose that some of the nascent mRNA transcripts in the *hpr1* Δ mutant are incomplete, being either aborted or degraded because of aberrant structures. These messages would not be exported to the cytoplasm. Thus, the increased transcription relative to that in the wild type does not result in increased message accumulation.

We believe that this structural explanation is plausible, given the synthetic lethality *hpr1* Δ displays when combined with either a *top1* Δ , a *top2*(Ts), or a *top3* Δ mutation (3) or with deletions in histones H3 and H4 (*hht1-hhf1* Δ) (14). Plasmids in yeast can become negatively supercoiled in a transcription-dependent manner when they are present in both *top1* Δ and *top1* Δ *top2*(Ts) mutant strains (4, 35). Indeed, plasmids can become negatively supercoiled in wild-type strains in a transcription-dependent manner given the proper conditions of a strong promoter on the plasmid producing a relatively long (7-kb) transcript (27). An elongating RNA polymerase complex generates an accumulation of positive supercoils in front of the complex and an accumulation of negative supercoils behind it (4, 23, 47). It is possible that in an *hpr1* Δ mutant, localized negative supercoiling may accumulate behind an elongating RNA polymerase II complex, which in turn would make the corresponding promoter more accessible.

We have previously reported that high-copy-number expression of the pre-mRNA splicing component *SUB2* suppresses *hpr1* Δ defects, including instability of plasmid pRM102 (15), and this has been confirmed by more recent studies (18). Our studies may give some insight into the mechanism of suppression. By Northern analysis, there is a three- to fourfold increase in the pRM102 *DED1* promoter transcript level in the *hpr1* Δ mutant carrying a high-copy-number *SUB2* plasmid, relative to that in the wild type. This suggests that high-copy-number *SUB2* does not suppress by returning transcription to a wild-type level. Rather, high-copy-number *SUB2* appears to make the defective transcripts into productive transcripts that can be exported to the cytoplasm. The increase in cytoplasmic message accumulation most likely reflects the increased transcription that occurs in *hpr1* Δ cells. In a mechanism that we do not understand, Sub2p overcomes the transcription defect of *hpr1* Δ cells, allowing production of full-length messages that can be processed and transported to the cytoplasm. We would

expect nuclear run-on analysis of *hpr1Δ* plus high-copy-number *SUB2* cells to show the same increase in RNA polymerase II density as *hpr1Δ* cells lacking the high-copy-number *SUB2* plasmid. However, we have not been able to do this experiment, as the run-on analysis requires hybridization to the *ori-amp* sequence and adjacent regions of the plasmid backbone that are also present on the *SUB2* plasmid. Nonetheless, we surmise that suppression occurs by a resolution of the mRNA processing-export defect of *hpr1Δ* transcription and not through a bypass of the transcription process. High-copy-number *Sub2p* could act as an RNA helicase to unwind inhibitory secondary structure that may be present in the nascent RNA molecule. Alternatively, *Sub2p* could act as an RNA-DNA helicase to unwind the RNA-DNA hybrid just behind RNA polymerase II, thereby allowing the RNA polymerase complex to regress and any blocking structure to be removed. However, in pre-mRNA splicing, *Sub2p* is not hypothesized to directly contact the RNA molecule. Rather, *Sub2p* contacts proteins that are bound to the 3' splice site region. *Sub2p* has been proposed to act as an RNase to aid in the removal of these proteins from the 3' splice site in a protein complex swap (19, 22, 36, 50). *Sub2p* may have a similar role in *hpr1Δ* suppression by aiding in the removal of a complex from the nascent RNA or the DNA template or the RNA-DNA hybrid region. It will be of interest to see if other *hpr1Δ* suppressors, in particular the recessive suppressors that are mutations in the basic transcription apparatus, act in a fashion similar to that of high-copy-number *Sub2p* by nuclear run-on studies.

We have proposed and tested several models of *hpr1Δ*-mediated plasmid instability. The aberrant transcription in an *hpr1Δ* strain does not pass through the plasmid centromere region and cause instability though missegregation. Sister chromatid recombination to form a dicentric plasmid also seems to be ruled out by our studies with recombination-deficient strains. We also found no evidence of plasmid sequestration in a membrane fraction. The most likely explanation is that the aberrant transcription and possible transiently altered plasmid supercoiling in some manner affects efficient replication at the yeast DNA replication *ARS* sequence on the plasmid. Once the transcription problem can be overcome, for example, by high-copy-number *SUB2*, even though the level of transcription remains high, plasmid stability is restored. This suggests that it is not the high transcription rate per se that affects origin firing but the defective transcription. Perhaps the presence of a trapped mRNA processing-export complex, for example, the TREX complex (41), or trapping of the plasmid in a mRNA export nuclear compartment occludes or prevents the DNA replication complex from assembling on the plasmid. In this way, a failure of DNA replication would account for the reduced plasmid stability and the low plasmid copy number in cells that have retained the plasmid.

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REFERENCES

1. Aguilera, A., and H. L. Klein. 1989. Genetic and molecular analysis of recombination events in *Saccharomyces cerevisiae* occurring in the presence of the hyper-recombination mutation *hpr1*. *Genetics* **122**:503–517.
2. Aguilera, A., and H. L. Klein. 1988. Genetic control of intrachromosomal

- recombination in *Saccharomyces cerevisiae*. I. Isolation and genetic characterization of hyper-recombination mutations. *Genetics* **119**:779–790.
3. Aguilera, A., and H. L. Klein. 1990. *HPRI*, a novel yeast gene that prevents intrachromosomal excision recombination, shows carboxy-terminal homology to the *Saccharomyces cerevisiae* *TOP1* gene. *Mol. Cell. Biol.* **10**:1439–1451.
4. Brill, S. J., and R. Sternglanz. 1988. Transcription-dependent DNA supercoiling in yeast DNA topoisomerase mutants. *Cell* **54**:403–411.
5. Caponigro, G., and R. Parker. 1996. Mechanisms and control of mRNA turnover in *Saccharomyces cerevisiae*. *Microbiol. Rev.* **60**:233–249.
6. Chang, M., D. French-Cornay, H. Y. Fan, H. Klein, C. L. Denis, and J. A. Jaehning. 1999. A complex containing RNA polymerase II, Paf1p, Cdc73p, Hpr1p, and Ccr4p plays a role in protein kinase C signaling. *Mol. Cell. Biol.* **19**:1056–1067.
7. Chavez, S., and A. Aguilera. 1997. The yeast *HPRI* gene has a functional role in transcriptional elongation that uncovers a novel source of genome instability. *Genes Dev.* **11**:3459–3470.
8. Chavez, S., T. Beilharz, A. G. Rondon, H. Erdjument-Bromage, P. Tempst, J. Q. Svejstrup, T. Lithgow, and A. Aguilera. 2000. A protein complex containing Tho2, Hpr1, Mft1 and a novel protein, Thp2, connects transcription elongation with mitotic recombination in *Saccharomyces cerevisiae*. *EMBO J.* **19**:5824–5834.
9. Chavez, S., M. Garcia-Rubio, F. Prado, and A. Aguilera. 2001. Hpr1 is preferentially required for transcription of either long or G+C-rich DNA sequences in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **21**:7054–7064.
10. Christianson, T. W., R. S. Sikorski, M. Dante, J. H. Shero, and P. Hieter. 1992. Multifunctional yeast high-copy-number shuttle vectors. *Gene* **110**:119–122.
11. Dyson, N. J. 1991. Immobilization of nucleic acids and hybridization analysis, p. 111–156. In T. A. Brown (ed.), *Essential molecular biology: a practical approach*, vol. 2. IRL Press at Oxford University Press, Oxford, United Kingdom.
12. Exinger, F., and F. Lacroute. 1992. 6-Azauracil inhibition of GTP biosynthesis in *Saccharomyces cerevisiae*. *Curr. Genet.* **22**:9–11.
13. Fan, H. Y., K. K. Cheng, and H. L. Klein. 1996. Mutations in the RNA polymerase II transcription machinery suppress the hyperrecombination mutant *hpr1Δ* of *Saccharomyces cerevisiae*. *Genetics* **142**:749–759.
14. Fan, H. Y., and H. L. Klein. 1994. Characterization of mutations that suppress the temperature-sensitive growth of the *hpr1Δ* mutant of *Saccharomyces cerevisiae*. *Genetics* **137**:945–956.
15. Fan, H. Y., R. J. Merker, and H. L. Klein. 2001. High-copy-number expression of *Sub2p*, a member of the RNA helicase superfamily, suppresses *hpr1*-mediated genomic instability. *Mol. Cell. Biol.* **21**:5459–5470.
16. Friedman, K. L., and B. J. Brewer. 1995. Analysis of replication intermediates by two-dimensional agarose gel electrophoresis. *Methods Enzymol.* **262**:613–627.
17. Hampsey, M. 1997. A review of phenotypes in *Saccharomyces cerevisiae*. *Yeast* **13**:1099–1133.
18. Jimeno, S., A. G. Rondon, R. Luna, and A. Aguilera. 2002. The yeast THO complex and mRNA export factors link RNA metabolism with transcription and genome instability. *EMBO J.* **21**:3526–3535.
19. Kistler, A. L., and C. Guthrie. 2001. Deletion of *MUD2*, the yeast homolog of U2AF65, can bypass the requirement for *sub2*, an essential spliceosomal ATPase. *Genes Dev.* **15**:42–49.
20. Kohrer, K., and H. Domdey. 1991. Preparation of high molecular weight RNA. *Methods Enzymol.* **194**:398–405.
21. Koshland, D., L. Rutledge, M. Fitzgerald-Hayes, and L. H. Hartwell. 1987. A genetic analysis of dicentric minichromosomes in *Saccharomyces cerevisiae*. *Cell* **48**:801–812.
22. Libri, D., N. Graziani, C. Saguez, and J. Boulay. 2001. Multiple roles for the yeast *SUB2p/UAP56* gene in splicing. *Genes Dev.* **15**:36–41.
23. Liu, L. F., and J. C. Wang. 1987. Supercoiling of the DNA template during transcription. *Proc. Natl. Acad. Sci. USA* **84**:7024–7027.
24. Lopez, M. C., M. Sanchez, E. Ferminan, and A. Dominguez. 1998. Disruption of six *Saccharomyces cerevisiae* genes from chromosome IV and basic phenotypic analysis of deletion mutants. *Yeast* **14**:1199–1208.
25. Mann, C., and R. W. Davis. 1983. Instability of dicentric plasmids in yeast. *Proc. Natl. Acad. Sci. USA* **80**:228–232.
26. Osborne, B. I., and L. Guarente. 1989. Mutational analysis of a yeast transcriptional terminator. *Proc. Natl. Acad. Sci. USA* **86**:4097–4101.
27. Osborne, B. I., and L. Guarente. 1988. Transcription by RNA polymerase II induces changes of DNA topology in yeast. *Genes Dev.* **2**:766–772.
28. Panzeri, L., L. Landonio, A. Stotz, and P. Philippsen. 1985. Role of conserved sequence elements in yeast centromere DNA. *EMBO J.* **4**:1867–1874.
29. Piruat, J. I., and A. Aguilera. 1996. Mutations in the yeast *SRB2* general transcription factor suppress *hpr1*-induced recombination and show defects in DNA repair. *Genetics* **143**:1533–1542.
30. Piruat, J. I., and A. Aguilera. 1998. A novel yeast gene, *THO2*, is involved in RNA pol II transcription and provides new evidence for transcriptional elongation-associated recombination. *EMBO J.* **17**:4859–4872.
31. Piruat, J. I., S. Chavez, and A. Aguilera. 1997. The yeast *HRS1* gene is

- involved in positive and negative regulation of transcription and shows genetic characteristics similar to *SIN4* and *GAL11*. *Genetics* **147**:1585–1594.
32. **Prado, F., J. I. Piruat, and A. Aguilera.** 1997. Recombination between DNA repeats in yeast *hpr1Δ* cells is linked to transcription elongation. *EMBO J.* **16**:2826–2835.
 33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 34. **Schneiter, R., C. E. Guerra, M. Lampl, G. Gogg, S. D. Kohlwein, and H. L. Klein.** 1999. The *Saccharomyces cerevisiae* hyperrecombination mutant *hpr1Δ* is synthetically lethal with two conditional alleles of the acetyl coenzyme A carboxylase gene and causes a defect in nuclear export of polyadenylated RNA. *Mol. Cell. Biol.* **19**:3415–3422.
 35. **Schultz, M. C., S. J. Brill, Q. Ju, R. Sternglanz, and R. H. Reeder.** 1992. Topoisomerases and yeast rRNA transcription: negative supercoiling stimulates initiation and topoisomerase activity is required for elongation. *Genes Dev.* **6**:1332–1341.
 36. **Schwer, B.** 2001. A new twist on RNA helicases: DExH/D box proteins as RNAPases. *Nat. Struct. Biol.* **8**:113–116.
 37. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. *Methods in yeast genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 38. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**:19–27.
 39. **Snyder, M., R. J. Sapolsky, and R. W. Davis.** 1988. Transcription interferes with elements important for chromosome maintenance in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **8**:2184–2194.
 40. **Strasser, K., and E. Hurt.** 2001. Splicing factor Sub2p is required for nuclear mRNA export through its interaction with Yra1p. *Nature* **413**:648–652.
 41. **Strasser, K., S. Masuda, P. Mason, J. Pfannstiel, M. Oppizzi, S. Rodriguez-Navarro, A. G. Rondon, A. Aguilera, K. Struhl, R. Reed, and E. Hurt.** 2002. TREX is a conserved complex coupling transcription with messenger RNA export. *Nature* **417**:304–308.
 42. **Struhl, K.** 1985. Nucleotide sequence and transcriptional mapping of the yeast *pet56-his3-ded1* gene region. *Nucleic Acids Res.* **13**:8587–8601.
 43. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**:60–89.
 44. **Tanaka, S., D. Halter, M. Livingstone-Zatchej, B. Reszel, and F. Thoma.** 1994. Transcription through the yeast origin of replication *ARS1* ends at the ABFI binding site and affects extrachromosomal maintenance of minichromosomes. *Nucleic Acids Res.* **22**:3904–3910.
 45. **Uemura, H., S. Pandit, Y. Jigami, and R. Sternglanz.** 1996. Mutations in *GCR3*, a gene involved in the expression of glycolytic genes in *Saccharomyces cerevisiae*, suppress the temperature-sensitive growth of *hpr1* mutants. *Genetics* **142**:1095–1103.
 46. **Warner, J. R.** 1991. Labeling of RNA and phosphoproteins in *Saccharomyces cerevisiae*. *Methods Enzymol.* **194**:423–428.
 47. **Wu, H. Y., S. H. Shyy, J. C. Wang, and L. F. Liu.** 1988. Transcription generates positively and negatively supercoiled domains in the template. *Cell* **53**:433–440.
 48. **Zaret, K. S., and F. Sherman.** 1982. DNA sequence required for efficient transcription termination in yeast. *Cell* **28**:563–573.
 49. **Zaret, K. S., and F. Sherman.** 1984. Mutationally altered 3' ends of yeast *CYC1* mRNA affect transcript stability and translational efficiency. *J. Mol. Biol.* **177**:107–135.
 50. **Zhang, M., and M. R. Green.** 2001. Identification and characterization of yUAP/Sub2p, a yeast homolog of the essential human pre-mRNA splicing factor hUAP56. *Genes Dev.* **15**:30–35.
 51. **Zhu, Y., C. L. Peterson, and M. F. Christman.** 1995. *HPRI* encodes a global positive regulator of transcription in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **15**:1698–1708.