DNA Replication Fork Pause Sites Dependent on Transcription

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Replication fork pause (RFP) sites transiently arresting replication fork movement were mapped to transfer RNA (tRNA) genes of *Saccharomyces cerevisiae* in vivo. RFP sites are polar, stalling replication forks only when they oppose the direction of tRNA transcription. Mutant tRNA genes defective in assembly of transcription initiation complexes and a temperature-sensitive RNA polymerase III mutant (*rpc160-41*) defective in initiation of transcription do not stall replication forks, suggesting that transcription is required for RFP activity.

In eukaryotes and prokaryotes the rate of DNA synthesis is at least five times the rate of RNA synthesis (*1*, *2*). Since simultaneous DNA and RNA synthesis can take place on the same part of a DNA molecule, collisions between DNA and RNA polymerases seem unavoidable. Analysis of collisions between *Escherichia coli* RNA polymerase and bacteriophage T4 or *E. coli* DNA polymerase have shown that replication forks pause when they encounter a transcription complex (*3*, *4*).

In *S. cerevisiae* two replication fork pause (RFP) sites have been found in systematic surveys of replication intermediates (RIs) of chromosome III (*5*). The RFP sites, which cause the accumulation of RIs of a particular size, were detected by two-dimensional gel electrophoresis as intense regions of hybridization along arcs of Y-shaped RIs (Fig. 1). One RFP site was downstream of Ty1-17 in a region expected to be replicated by a replication fork initiated at *ARS307* (Fig. 1). In that Ty elements are actively transcribed (*6*), this RFP might be the result of a head-on collision between the replication and transcription machineries.

Analysis of RIs of overlapping fragments from the region containing Ty1-17 revealed the RFP as an oblong region of intense hybridization (Fig. 1C). The size and oblong shape of this region indicate that replication forks pause throughout a region of approximately 500 base pairs (bp) rather than at a specific point. The RFP appeared to include the downstream, long terminal repeat (3′ LTR) of Ty1-17 and the *SUP53* tRNA gene, and its position on the Y arc suggested that forks moving leftward from *ARS307* were stalling. To confirm this observation and to determine whether forks moving rightward through the region also stalled, we inactivated replication origins flanking the RFP site. Deletion of *ARS306* caused the region containing the RFP to be replicated exclusively by forks moving leftward from *ARS307* and had no effect on RFP activity (Fig. 1D). In contrast, inactivation of *ARS307* caused this region to be replicated predominantly by forks moving rightward from *ARS306* and severely diminished RFP activity (Fig. 1E). These results suggest that the RFP is polar and affects only the leftward-moving forks.

The RFP does not depend on an intact Ty element as the RFP was present in a strain in which an intrachromosomal LTR-LTR recombination left a solo LTR in place of Ty1-17 (*6a*). Moreover, an RFP was mapped to a solo LTR and a $tRNA^{Glu}$ gene located approximately 2 kb left of Ty1-17 (Fig. 1F). The absence of this RFP in a strain from which *ARS306* was deleted demonstrates that it is forks moving rightward from *ARS306* that stall (Fig. 1G). A solo LTR and a $tRNA^{Arg}$ gene on chromosome X (*7*) replicated by a leftward moving fork from *ARS5* were also associated with an RFP site (*6a*). Thus, the common features of these RFPs include an LTR and a tRNA gene whose transcription opposes the approaching replication fork.

To further characterize the RFP, we cloned a 1.5-kb fragment containing the Ty1-17 LTR and *SUP53* in both orientations in plasmid pAT1, which was maintained at one to two copies per cell (Fig. 2A). An RFP was present when the orientation of the LTR and the direction of transcription of *SUP53* opposed the replication forks approaching them (head-on orientation, Fig. 2B), but was absent in the opposite (codirectional) orientation (Fig. 2C). The fraction of RIs in the plasmid RFP (Table 1, line 1) indicated that it was similar in strength to the chromosomal RFP. The polar nature of the RFP is demonstrated by the similar fractions of RIs present in the region containing the LTR and *SUP53* in a codirectional orientation and a region of similar size in the vector alone (Table 1, lines 2 and 3).

Analysis of subclones demonstrated that

Table 1. Quantitative analysis of RFP sites. Plasmid constructs contain fragments indicated cloned at the Bam HI site of pAT1 (*21*). RIs of plasmid fragments of 3.0 to 3.5 kb carrying the tRNA gene or LTR within the middle half were analyzed by two-dimensional gel electrophoresis. The fraction of total replication intermediates in the RFP site was determined by either a Molecular Dynamics computing densitometer (model 300B) or Molecular Dynamics PhosphorImager (model 445 SI) with ImageQuant software. All constructs were analyzed in at least two independent DNA preparations, and the values presented represent the mean and standard deviation of the measurements. All inserts were also examined in the codirectional orientation in pAT1. None of the codirectional constructs showed any evidence of an RFP. Transcription efficiencies of *SUP53*-**a** were measured by suppression of the *lys2-801* amber allele and quantitated as the fraction of plasmid-bearing cells able to grow without lysine (*11*). ++++, most *SUP53* cells grew without lysine; +, about 1 in 10^4 *SUP53* cells grew. Transcription efficiencies of *SUP2* are taken from (*12*). +++, abundant transcript present, −, no transcript detected.

Plasmid construct	Replication intermediates in RFP (%)	Transcription efficiency
pAT15B (Fig. 2): 1.5-kb Bgl II–Eco RI fragment carrying 3′ LTR and *SUP53*, head-on orientation	28.0 ± 5.2	NA
pAT16B (Fig. 2): as pAT15B but codirectional orientation	5.5 ± 0.2	NA
pAT1, vector alone	6.3 ± 0.7	NA
pAT20B: 780-bp Rsa I fragment carrying *SUP53*, head-on	44.7 ± 2.7	NA
pAT18B: 370-bp Bgl II–Rsa I fragment carrying 3′ LTR, head-on	3.1 ± 0.1	NA
pASC12B: 762-bp fragment carrying *SUP11-1* (*22*), head-on	11.9 ± 1.0	NA
pAT21B (*10*): 240-bp *SUP53* fragment, 60 bp upstream and 46 bp downstream flanking sequence, head-on	18.7 ± 1.8	++++
pAT26B (*10*): 212-bp *SUP53* fragment, 60 bp upstream and 7 bp downstream flanking sequence, head-on	20.7 ± 0.8	++++
pAT28B (*10*): 187-bp *SUP53* fragment, 7 bp upstream and 46 bp downstream flanking sequence, head-on	9.8 ± 1.0	+
pAT24B (*10*): 159-bp *SUP53* fragment, 7 bp upstream and 18 bp downstream flanking sequence, head-on	7.1 ± 0.9	+
pAT356B (*23*): 1-kb Eco RI–Bam HI *SUP2*-**o** fragment, head-on	12.8 ± 1.3	+++
pAT565B (*23*): 1-kb Eco RI–Bam HI *sup2*-**o** (C56G), head-on	3.0 ± 0.5	−

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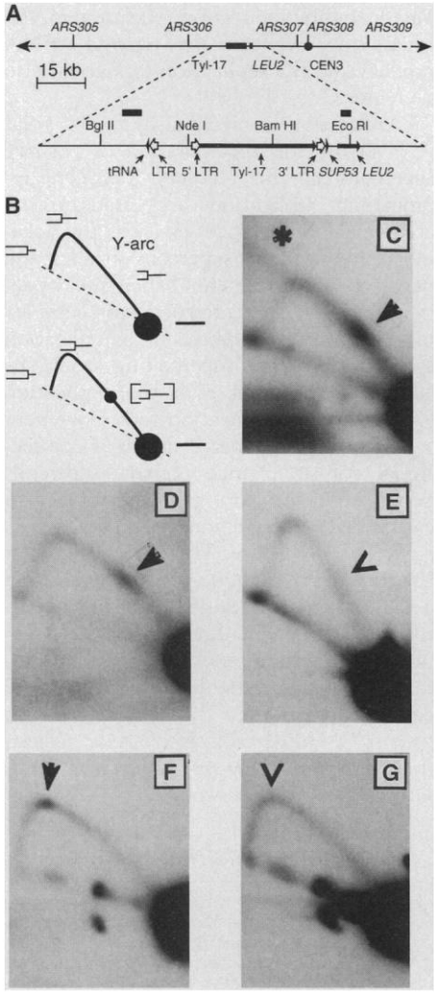
a fragment carrying *SUP53* alone contained an RFP site (Table 1, line 4), while a fragment containing the LTR was devoid of RFP sites in either orientation (Table 1, line 5). A polar RFP also mapped to the *SUP11-1* $tRNA^{Tyr}$ gene, which in its natural context is not associated with an LTR (Table 1, line 6). Moreover, a polar RFP site on chromosome V is associated with a $tRNA^{Ile}$ gene (8). Thus, it appears to be a general property of tRNA genes to cause polar RFPs.

Transcription complex assembly on tRNA genes occurs in three steps (9). First, transcription factor TFIIIC binds to the intragenic promoter elements, box A and box B. The TFIIIC then promotes binding of TFIIIB to a region upstream of the transcription start site. The TFIIIB-DNA complex finally recruits RNA polymerase III (RNAPIII). Deletion of sequences upstream of the transcription start site diminishes or abolishes transcription in vitro (9). Other than transcription termination, no specific function has been attributed to sequences immediately downstream of yeast tRNA genes (9).



To examine the role of sequences flanking *SUP53* in RFP activity and transcription, we cloned (in pAT1) fragments containing the tRNA gene flanked by different amounts of sequences upstream of the transcription start site and downstream of the transcription termination site (*10*). A 240-bp fragment containing the 134-bp *SUP53* gene flanked by 60 bp of upstream and 46 bp of downstream sequence resulted in an RFP comparable in strength to the chromosomal RFP (Table 1, line 7). Deletion of a 28-bp sequence flanking the *SUP53* transcription termination site had no significant effect on RFP activity (Table 1, line 8). In contrast, deletion of a 53-bp sequence flanking the transcription start site caused a dramatic reduction in RFP activity (Table 1, lines 9 and 10). Therefore, the tRNA gene along with the 60-bp sequence immediately upstream of the transcription start site is sufficient for full RFP activity in this plasmid context.

Transcriptional efficiencies of these *SUP53* constructs were determined by their abilities to suppress a *lys2-801* amber allele (*11*). Plasmids carrying *SUP53-a* flanked by 60 bp of upstream sequence suppressed efficiently (Table 1). In contrast, plasmids carrying the tRNA gene flanked by only 7 bp of upstream sequence supported growth without lysine inefficiently, demonstrating that deletion of sequences immediately upstream of the transcription start site severely diminishes *SUP53* transcription (Table 1). This correlation of RFP activity of *SUP53* with its transcriptional activity could reflect a causal relation. Alternatively, a DNA structure within the upstream sequences could be difficult for the replication apparatus to unwind.

To distinguish between these possibilities, the effect on RFP activity of a point mutation in box B of the *SUP2* $tRNA^{Tyr}$ gene was examined (Table 1, lines 11 and

Fig. 1. Regions containing a long terminal repeat (LTR) and a tRNA gene act as polar RFP sites. (**A**) The central region of chromosome III, showing the positions of ARS elements, genes and the centromere, *CEN3*. *ARS308* is an inefficient chromosomal origin of replication whereas the other *ARS* elements indicated are efficient (*5*). The region used to examine chromosomal RFP sites has been enlarged. Filled arrows indicate directions of transcription and open arrows show orientations of LTR elements. Pertinent restriction sites are shown. The black rectangular boxes indicate probes, and the bar represents 15-kb pairs. (**B**) Patterns expected. Square brackets indicate accumulated replication intermediates of a particular molecular mass, which are manifested as a spot of intense hybridization (small black circle) along an otherwise uniform arc of Y-shaped replication intermediates (Y arc). The dashed line indicates the arc of linear molecules. Nonreplicating DNA fragments are indicated as a big black circle. (C, D, and E) Analysis of a 3.5-kb Bam HI–Eco RI fragment containing the centromere-proximal half of Ty1-17 and *SUP53* from strains (**C**) YNN214 (*19*), (**D**) YAΔ306 (*19*), and (**E**) YP528 (*19*). The arc of double Y-shaped replication termination intermediates [marked by * in (C)] results from forks emanating from *ARS306* and *ARS307* meeting within the fragment analyzed. The slight accumulation of replication intermediates along the ascending part of the Y arc in (E) probably resulted from forks moving leftward from *ARS308* or *ARS309* and stalling at the RFP site. (F and G) Analysis of the 4-kb Bgl II–Nde I fragment containing a $tRNA^{Glu}$ gene and a solo LTR in strains (**F**) YNN214 and (**G**) YAΔ306. DNA preparation, two-dimensional gel analysis, blotting, and hybridization procedures have been described (*20*). Locations of RFP sites are indicated by a filled arrowhead. The caret in Fig. 1, E and G, indicates the expected position of an RFP site. Spots along the arc of linear molecules are partial digestion products of genomic DNA or cross-hybridizing chromosomal fragments.

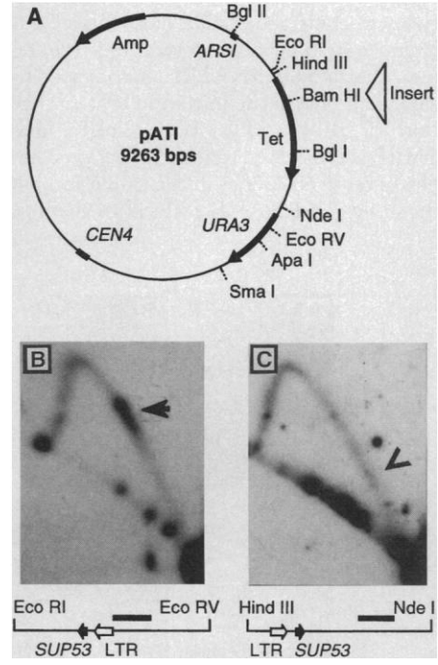


Fig. 2. A polar RFP site functions in a plasmid. (**A**) A map of pAT1 (*21*) showing restriction sites pertinent to this study. Bgl II linkers were added to restriction fragments of interest, and the fragments cloned in the single Bam HI site of pAT1 to create the plasmids used. The 554-bp Bam HI–Bgl I fragment from the tetracycline gene was used as the hybridization probe. (**B**) Two-dimensional gel analysis of replication intermediates of the 3-kb Eco RI–Eco RV fragment of pAT15B, which carries the 1.5-kb Bgl II–Eco RI fragment containing the 3′ LTR of Ty1-17 and the *SUP53* gene cloned in the head-on orientation with respect to replication forks from *ARS1*. (**C**) Analysis of the 3.1-kb Hind III–Nde I fragment of pAT16B, which carries the same fragment as pAT15B in the codirectional orientation. Maps of the restriction fragments used for two-dimensional gel analysis are shown below the panels. Symbols are as described in Fig. 1.

12). This mutation abolished transcription by preventing binding of TFIIIC (*12*). The wild-type *SUP2* gene caused a polar RFP. In contrast, the *sup2* mutant showed no RFP activity, demonstrating that inactivation of transcription abolishes RFP activity. Therefore, a conserved structure within upstream sequences is unlikely to play a role in RFP activity.

To determine whether the formation of the initiation complex on a tRNA gene was sufficient for RFP activity or whether binding of RNAPIII was also necessary, we examined the effect on RFP activity of a temperature-sensitive (ts) mutation (*rpc160-41*) in the largest subunit of RNAPIII. This mutation caused a fivefold reduction in the abundance of tRNA at the permissive temperature and an additional fourfold decrease at the nonpermissive temperature in vivo; the abundance of 5S ribosomal RNA (rRNA) is not affected at either temperature (*13*). Since 5S rRNA and tRNA are stable in mutant cells, the differences in their abundance must reflect differences in their rate of synthesis. Therefore, the mutant RNAPIII appears specifically defective in the initiation of transcription of tRNA (*13*). Both TFIIIC and TFIIIB are expected to form initiation complexes on tRNA genes in the ts mutant, but the ability of RNAPIII to bind to them or initiate transcription is reduced.

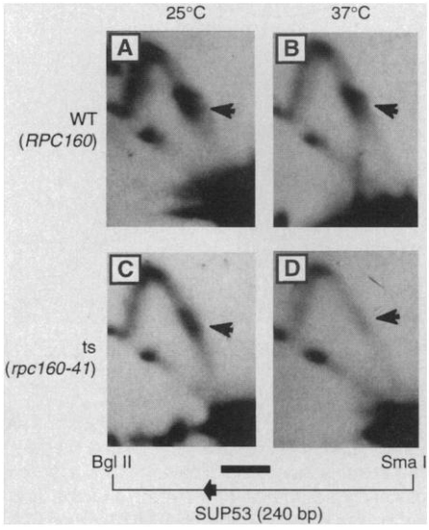


Fig. 3. Activity of RNAPIII is required for RFP activity. Strains YNN281-21B and ts41-21B (*19*) were grown at the permissive temperature (25°C) to mid-log phase and shifted to nonpermissive temperature (37°C). Cells were harvested 2 and 4 hours after the shift. Control cultures were maintained at 25°C. Panels show the analysis of the 3.4-kb Bgl II–Sma I fragment of pAT21B carrying *SUP53*. (**A**) YNN281-21B grown at 25°C. (**B**) YNN281-21B, 2 hours at 37°C. (**C**) ts41-21B grown at 25°C. (**D**) ts41-21B, 2 hours at 37°C. Samples taken 4 hours after the temperature shift gave similar results. The map of the restriction fragment analyzed is shown at the bottom of the figure. Symbols are defined in Fig. 1.

The RFP activity of *SUP53* was tested in the *rpc160-41* strain and its wild-type parent. *SUP53* had efficient RFP activity at both 25°C and at 37°C in the wild-type parental strain (Fig. 3, A and B). RFP activity was reduced twofold in the mutant strain at the permissive temperature (Fig. 3C), and eightfold following a shift to the nonpermissive temperature, 37°C (Fig. 3D). These reductions in RFP activity are in good agreement with the reduction in tRNA accumulation observed in vivo (*13*). These observations suggest that formation of an initiation complex is not sufficient for RFP activity, and that binding of RNAPIII to the initiation complex on the tRNA gene and possibly transcription per se is necessary.

The correlation observed between RFP activity and transcriptional activity of the tRNA genes examined suggests that transcription transiently blocks replication fork movement and that tRNA genes are transcribed and replicated concurrently. The polar nature of the pause, demonstrated by the failure of tRNA genes in codirectional orientations to cause RFPs, indicates that only head-on collisions between the transcription and replication machinery result in significant RFP activity.

The polar replication fork barriers (RFBs) located downstream of 35S rRNA genes in *S. cerevisiae* (*14*) prevent head-on collisions between the replication and transcription machineries. However, dependence of tRNA-associated RFP activity on transcription distinguishes it from the RFB, which is independent of transcription (*14*). It is not yet certain whether RFBs or RFP sites are associated with other actively transcribed genes. There is no evidence of an in vivo RFP site associated with 5S rDNA genes of *S. cerevisiae* (*14*). Similarly, a 5S rRNA gene cloned in pAT1 failed to stall replication forks (*6a*). It is possible that the 5S rRNA gene-specific transcription factor, TFIIIA (*15*), facilitates the passage of replication forks through 5S rRNA genes. However, it is not clear what fraction of 5S rRNA genes are transcribed in vivo, and it is possible that the 5S rRNA gene cloned into pAT1 was not transcribed.

The fraction of RIs within the RFP region ranged from 12 to 45% for the wild-type tRNAs examined and presumably reflects their transcriptional efficiencies. These values can be used to estimate the duration of the pause (*16*). To account for an accumulation of 20% of RIs within the RFP region, every fork traversing the fragment would have to pause for approximately 10 s, an interval two to three times longer than the interval required for RNAPIII to transit a tRNA gene (*2*). If tRNA genes are not continuously transcribed, then only the replication forks that encounter an active transcription complex might pause, and the length of the required pause would increase. If the duration of the pause approached the interval required for a plasmid to be replicated by a single fork (100 s for pAT1), replication termination intermediates should be apparent in the plasmid RIs that we examined. The absence of such intermediates sets an upper limit on the length of the pause.

The polar RFP site identified by these studies could result from a direct interaction between the transcription and replication apparatuses. Except for the initial binding of transcription factors, RNA synthesis occurs on one strand of the DNA template. DNA replication occurs concurrently on both strands of the DNA template, but some replication factors are asymmetrically distributed between the two strands of the fork (*17*). Studies on the minimal in vitro T4 replication system provide support for interactions between proteins on the leading strand of the replication fork and the transcription complex moving in the opposite direction on the complementary strand (*4*). The observed stalling of RNAPIII at the transcription termination site of a tRNA gene in vitro (*2*) could be responsible for stalling replication forks in vivo. An interaction between the nascent tRNA product and the replication fork could also be required for the RFP.

An alternative mechanism for the polar RFP site is the accumulation of positive supercoils in the template between approaching replication and transcription complexes. Both processes cause the accumulation of positive supercoils ahead of and negative supercoils behind their sites of action (*1, 18*). When a transcription complex moves toward an approaching replication fork, this positive supercoiling would be additive and might stall the replication fork. However, replication fork movement would not be hampered during a codirectional collision, since positive supercoils generated in front of a replication fork will cancel negative supercoils generated behind an RNA polymerase. The observation that the RFP site spans 500 bp and is larger than a tRNA gene is more easily explained by this model than by direct interactions between replication and transcription proteins. Moreover, the likelihood that the pause is longer than the 3- to 5-s transit time of RNAPIII on a tRNA gene suggests that the approaching replication fork might slow transcription as well.

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10. The 240-bp, 212-bp, 187-bp, and 159-bp fragments containing *SUP53* or *SUP53-a* flanked by different amounts of sequences upstream of the transcription start site and downstream of the transcription termination site were amplified by polymerase chain reaction from plasmid pAT20 or YEP13-SUP53 (obtained from D. Engelke). Primers A (5′-CGCT**GGATCC**TCCTTGTTCATGTGTGTTC-3′) and B (5′-CGCT**GGATCC**TTTCTCAACAAGTAATTGG-3′) became annealed 60 bp and 7 bp upstream of the transcription start site, respectively. Primers C (5′-CGCT**GGATCC**TTGATTCTGTGCGATAGCG-3′) and D (5′-CGCT**GGATCC**GTTCTCGTTATGTTGAGG-3′) became annealed 46 bp and 18 bp downstream of the transcription termination site, respectively. Primers were designed to create Bam HI site at the ends (boldface letters). Amplified fragments were then cloned in both orientations in pAT1 (*21*).
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19. YNN214 is *MAT***a**, *ura3-52, lys2-801, ade2-101* [R. Sikorski and P. Hieter, *Genetics* **122**, 19 (1989)]. YAΔ306 is YNN214 carrying a deletion of *ARS306* constructed as described (*20*). YP528 is YNN214 carrying a T to G point mutation at position 9 of the ARS consensus sequence of *ARS307* (*20*). YP306D is *MAT*α, *ura3-52, lys2-801, ade2-101* carrying a deletion of *ARS306* constructed as described (*20*). YNN281 is *MAT***a**, *ura3-52, lys2-801, ade2-101, his3-Δ200, trp1-901*. The ts41 strain is YNN281 carrying the *rpc160-41* mutation (*13*). Both YNN281 and ts41 were transformed with plasmid pAT21B (Table 1) to create YNN281-21B and ts41-21B, respectively. Media used were as described [F. Sherman, *Methods Enzymol.* **194**, 3 (1991)].
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