

Video Article

# Direct Restart of a Replication Fork Stalled by a Head-On RNA Polymerase

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## Abstract

*In vivo* studies suggest that replication forks are arrested due to encounters with head-on transcription complexes. Yet, the fate of the replisome and RNA polymerase (RNAP) following a head-on collision is unknown. Here, we find that the *E. coli* replisome stalls upon collision with a head-on transcription complex, but instead of collapsing, the replication fork remains highly stable and eventually resumes elongation after displacing the RNAP from DNA. We also find that the transcription-repair coupling factor, Mfd, promotes direct restart of the fork following the collision by facilitating displacement of the RNAP. These findings demonstrate the intrinsic stability of the replication apparatus and a novel role for the transcription-coupled repair pathway in promoting replication past a RNAP block.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/1919/>

## Protocol

This method was used in the research reported in [Pomerantz and O'Donnell, Science 327, 590-592 \(2010\)](#).

1. Assembly of a halted RNAP elongation complex was performed as follows:

500 nM final concentration of *E. coli* RNAP  $\sigma^{70}$  HE was mixed with 5 nM final concentration of a biotinylated 3.6 kb DNA template containing the T7A1 promoter in 100  $\mu$ l of buffer A (20 mM Tris-Cl (pH 7.5), 8 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 5 mM DTT, 10% glycerol) for 10 min at 37 °C. 100  $\mu$ M of ApU and 40  $\mu$ M each of GTP, ATP, and CTP were added for an additional 10 min at 37 °C.

2. Immobilization of the halted RNAP elongation complex onto streptavidin beads was performed as follows:

200  $\mu$ l of streptavidin magnetic coated beads (Invitrogen) were added to the reaction above for 10 min at room temp.

3. Purification of the immobilized halted RNAP elongation complex was performed as follows:

The beads (from above) were washed 5 times with 0.9 ml of buffer A containing 0.75 M NaCl, 200  $\mu$ g/ml heparin, and 20  $\mu$ g/ml ssDNA (The supernatant was removed after each wash step by magnetic separation.) Next, the beads were washed 2 times with 0.9 ml of buffer A.

4. Formation of a replication fork downstream relative to the head-on RNAP was performed as follows:

The beads (from above) were resuspended in 100  $\mu$ l of New England Biolabs buffer 4 and 10 units of Sap I (New England Biolabs) was added for 10 min at 37 °C. The beads were washed 3 times with 0.9 ml of buffer A and then resuspended in 50  $\mu$ l of Quick T4 Ligation reaction buffer (New England Biolabs). 2  $\mu$ l of Quick T4 ligase (New England Biolabs) was added along with 6 nM final concentration of pre-annealed forked DNA (RP10, RP22, RP25) for 10 min at room temp. The beads were washed 3 times with 0.9 ml of buffer A.

5. Assembly of the replisome and initiation of leading strand synthesis at the replication fork was performed as follows:

448 pmol of DnaB (as hexamer) was incubated with the beads 150  $\mu$ l of buffer A (20 mM Tris-Cl (pH 7.5), 8 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 5 mM DTT, 10% glycerol) for 30 s at 23 °C. 4.9 pmol of Pol III\* (Pol III HE minus  $\beta$ ), 15 pmol of  $\beta$ , 2 mM ATP, and 60  $\mu$ M each of dGTP and dATP were added to a volume of 200  $\mu$ l and incubated a further 5 min at 37 °C. Replication was initiated upon adding 10  $\mu$ g SSB and 24  $\mu$ M each of dATP and dTTP along with [ $\alpha$ -<sup>32</sup>P]dTTP and [ $\alpha$ -<sup>32</sup>P]dCTP (specific activity, 3,000-5,000 cpm/pmol) to a final volume of 250  $\mu$ l. Reactions were terminated after 10 min upon adding 12  $\mu$ l of 500 mM EDTA.

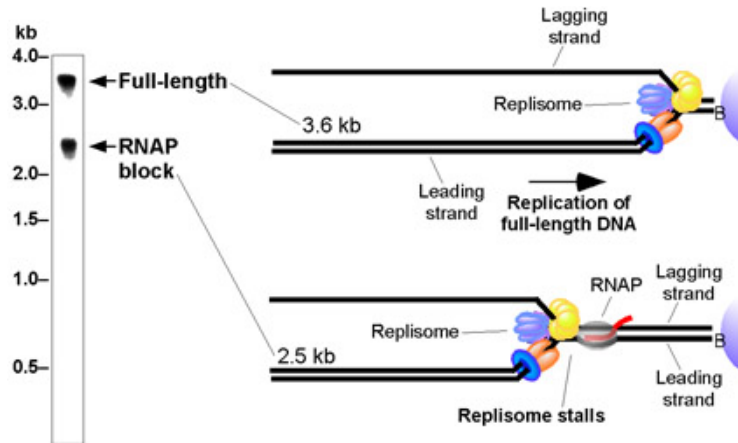
6. Purification and concentration of the radio-labeled DNA products was performed as follows:

The beads (from above) were boiled after the reaction was terminated in order to remove the DNA from the beads. Any residual DNA remaining attached to the beads was removed by treating the beads with proteinase K in a volume of 10  $\mu$ l for 30 min at 50 °C. The beads were then boiled and the supernatant was removed by magnetic separation. The combined supernatant containing the DNA was purified using the Qiagen PCR Cleanup kit. Purified radio-labeled DNA products were then analyzed in an alkaline agarose gel.

\*Replication in the absence of a head-on RNAP halted elongation complex was performed as above, however,  $\sigma^{70}$  was omitted.

### Representative Results:

Collision of the replisome with a head-on RNAP usually results in two products of 2.5 kb and 3.6 kb in length. The 2.5 kb product represents the length of the DNA from the fork to the halted RNAP and results from replisome stalling upon collision with the RNAP. The 3.6 kb product represent full-length DNA and results from either incomplete occupancy of the promoter by RNAP or partial replisome read-through of the head-on RNAP. A good or accurate result demonstrates that approximately 50% full-length DNA is produced. However, in some cases less occupancy of the promoter by RNAP occurs and a higher percentage of full-length DNA is observed since a greater population of replisomes do not encounter a head-on RNAP. Replication in the absence of a halted RNAP results in only full-length DNA (3.6 kb).



**Figure 1 The replisome is impeded by a head-on RNAP.**

**(A)** Experimental setup. A *E. coli* RNAP halted elongation complex was assembled on a biotinylated DNA containing the T7A1 promoter as previously described (9). Briefly, a halted elongation complex was formed 20 bps downstream from the promoter by incubating RNAP holoenzyme with biotinylated DNA for 10 min followed by the addition of ApU, GTP, ATP, and CTP for a further 10 min. Streptavidin beads were added for an additional 10 min then the beads were washed five times each with 0.9 ml of buffer containing 0.75M NaCl, 200 mg/ml heparin, and 100 nM single-strand DNA to remove unstable RNAP-DNA complexes and excess NTPs. The DNA was then digested with SapI, washed, and ligated to a pre-annealed forked DNA. The beads were washed again prior to assembly of the replisome. The promoter is located 2.5 kb from the fork and is oriented to direct transcription towards the fork. **(B)** DnaB was added then the replisome was assembled and replication was initiated either in the presence (lane 2) or absence (lane 1) of a head-on RNAP.

## Disclosures

Washing the halted elongation complex bound to the beads with high salt is critical in order to remove any unstable or non-specifically bound RNAP molecules. RNAP has an extremely high affinity for primer-template type DNA structures. Thus, it is necessary to remove such non-specific RNAP-DNA complexes in order to perform replication from the primer-template. Concentrating the radio-labeled DNA products using the Qiagen PCR purification kit is also critical in order to obtain a high enough radioactive signal that can be observed in the gel.

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