

PERSPECTIVE

Replication fork pausing and recombination or “gimme a break”

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Orderly replication of DNA is a prerequisite to the faithful segregation of the chromosomes before cell division. These processes occur in an environment where the natural order of events can be perturbed by DNA damage, which may in turn cause the replication fork to stall or even break down. Such events can trigger cellular checkpoints, which allow time for repair of damage before replication resumes. It is interesting that replication pausing can also occur naturally and that pausing and specific pause sites have been conserved throughout evolution in both prokaryotes and eukaryotes. Although it appears that these sites are important for the regulation of replication termination and to prevent collisions between the replication and transcription machinery, there is no definitive evidence as to their precise function. In this review we consider replication pausing in prokaryotes and eukaryotes. We distinguish between replication blocks and pause sites as well as programmed versus accidental pausing. We also discuss how pausing can lead to increased recombination.

Programmed replication pauses

Natural pause sites in bacteria

The bidirectional replication of the *Escherichia coli* chromosome starts at the origin (at 84 min) and ends in the diametrically opposed terminus region (Fig. 1). The terminus region is flanked by specific nucleotide sequences, the *Ter* sites, that are bound by the protein Tus (for review, see Hill 1996). The *Ter*–Tus complex blocks the progression of replication forks in a polar manner by inhibiting the unwinding action of replicative helicases (Sahoo et al. 1995). The complex forms a replication fork trap in which the forks can enter but from which they cannot exit. Although they are called terminators, fork movement beyond these sites can be detected under certain circumstances, suggesting that they are not an absolute barrier to replication and rather act as pause sites. Three of the six *Ter* sites are located between 23 and 28

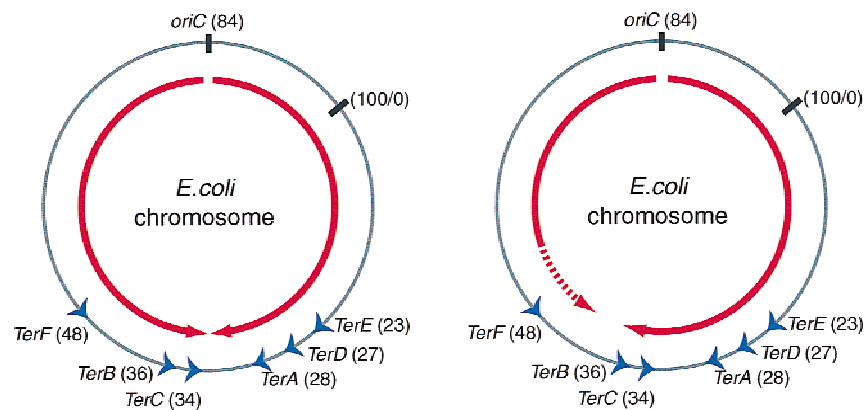
min and the others between 34 and 48 min, comprising a 1200-kb region. The *Ter* sites are oriented such that pausing occurs only if one of the two forks passes the natural merge point, which has been determined by labeling experiments in synchronized cultures to map at 31.2 min (Bouche et al. 1982). Thus, it is likely that most often the two forks do not meet systematically at a *Ter* site but merge naturally. This may explain why Tus mutants, which eliminate pausing at *Ter* sites, exhibit a deleterious phenotype only when chromosome replication has been made asymmetrical and therefore terminates in an inappropriate region (Dasgupta et al. 1991; Hill 1996). Several *E. coli* plasmids also carry replication terminators (for review, see Hill 1996). In the plasmid R1, inactivation of the terminus leads to plasmid instability and a change in the replication mode to generate a rolling circle. It was proposed that in the absence of the terminator, the 3′ end of the leading strand displaces the 5′ end of the previously synthesized DNA (Krabbe et al. 1997). This does not seem to occur for the *E. coli* chromosome in *tus* mutants.

Although the coordination of chromosome replication and cell cycle is mainly at the level of replication initiation, the replication terminus also has a role, as it contains elements responsible for the connection of replication termination and cell division (Corre et al. 1997; Steiner and Kuempel 1998). The terminus region of *E. coli* is the site of decatenation of circular chromosomes by topoisomerase IV (Zechiedrich and Cozzarelli 1995). In *Bacillus subtilis*, chromosomal replication is arrested at a specific terminator site by the binding of a replication terminator protein (RTP), which inhibits the action of the replicative helicases (for review, see Bussiere and Bastia 1999). Under certain stress conditions, such as the induction of the stringent response by amino acid starvation, *Ter*-like sites located ~200 kb from the origin are activated by RTP binding. These highly regulated origin-proximal replication arrest sites act as an additional level of replication control (Autret et al. 1999). Transcription progressing in the orientation permissive for replication dislodges RTPs from the DNA (Mohanty et al. 1998; Bussiere and Bastia 1999), which may participate in the control of replication termination.

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Figure 1. The *E. coli* chromosome, the position of *oriC* and the six *Ter* sites are shown (Kamada et al. 1996). The red arrows represent the direction of DNA replication initiated from *oriC*. (Left) The two forks approach the terminus region at approximately the same time having passed unimpeded through the *Ter* sites; (right) the counterclockwise replication fork encountered a replication block and restarted replication (broken red line). As a result, the clockwise replication fork enters into the terminator region and, in this example, pauses briefly at *TerC*, continues to *TerB*, and again pauses to await the arrival of the counterclockwise fork.



Ter sites have a role in the maintenance of genome integrity by limiting the encounter of the two replication forks to a defined region of the chromosome. Nevertheless, arrest of the replication fork leads to the formation of a Y structure, with a single-stranded gapped region and DNA ends from the newly replicated strands at the fork junction, any of which can be substrates for DNA recombination enzymes. Consequently, *Ter* sites have the potential to stimulate homologous recombination and thus compromise genome integrity.

Replication pause at Ter induces homologous recombination

The first example of a link between replication arrest at *Ter* and homologous recombination was described by the Horiuchi laboratory (Horiuchi et al. 1994). Under RNase H-defective conditions (e.g., an *rnh* mutation), replication can be initiated in *E. coli* from sites that are distinct from the origin (Kogoma 1997). In an attempt to isolate new replication origins activated in an *rnh* mutant, Horiuchi and collaborators ligated a bank of *E. coli* chromosomal fragments to an antibiotic marker. They identified eight sequences that allowed the maintenance of an origin-less plasmid. Interestingly, these sequences did not allow autonomous replication but, rather, acted as recombination hot spots allowing maintenance of the free plasmid molecules by their ability to permit frequent recombination into and out of the chromosome. Seven of eight “hot” sequences are located in the terminus region of the chromosome, and the hyper-recombination property of three of these sequences depends on the presence of the Tus protein and, hence, on the blockage of the replication forks by the Tus–*Ter* complex. For one hot sequence, hyper-recombination was dependent on the presence of *chi*, a specific octameric sequence recognized by RecBCD, the complex that repairs DNA double strand breaks (DSBs) in *E. coli* (Myers and Stahl 1994). The requirement for a *chi* site on the hot DNA indicated that the recombination events that allowed the maintenance of origin-less plasmids were initiated by RecBCD. Because RecBCD only acts on double-stranded DNA ends, it was proposed that in the *rnh* mutant, in

which replication initiates from sites other than the origin, the arrest of replication forks at *Ter* leads to the formation of DSBs (Horiuchi et al. 1994).

Hyper-recombination at *Ter* was studied further by introducing an ectopic *Ter* site in the *lacZ* region of the *E. coli* chromosome (Horiuchi and Fujimura 1995). In this *lacZ::Ter* strain, the clockwise replication fork was blocked halfway, whereas the counterclockwise replication fork was arrested in the terminus region at the natural *Ter* sites. This study showed that the hyper-recombination activity in the vicinity of *Ter* was not dependent on the *rnh* background, as it could be observed at this ectopic *Ter* site. Interestingly, the viability of cells in which *lacZ::Ter* was active was decreased by the inactivation of homologous recombination proteins, RecA and RecBC. Furthermore, the SOS response, the expression of a set of genes involved in DNA repair, was induced in the blocked strains. A model was proposed in which the blocked replication forks were broken and repaired by homologous recombination catalyzed by RecBCD and RecA (Horiuchi and Fujimura 1995). In cells defective for homologous recombination, the absence of repair would cause cell death. SOS induction would ensure that the cells are given the time to repair the broken replication forks prior to cell division.

In an independent study of the consequences of replication fork blockage, inverted *Ter* sites were inserted into the terminus region of the chromosome so that both replication forks were blocked, 2 kb apart. The viability of this *Inv–Ter* strain was dependent on SOS induction (Sharma and Hill 1995) and RecBCD-mediated homologous recombination (Sharma and Hill 1995; Michel et al. 1997). This suggested that the arrested replication forks were broken, as in *lacZ::Ter* strains. Interestingly, a single round of replication was not sufficient for SOS induction in the *Inv–Ter* strain (Sharma and Hill 1995). Because a stalled replication fork alone may be insufficient to induce the SOS response (Sassanfar and Roberts 1991), it was proposed that the inducing signal, breakage, was provided by a second replication fork. The first forks would remain arrested for a sufficient period of time to allow a second round of replication to arrive. Two scenarios were envisaged: Either the second round of repli-

cation forks utilize the newly synthesized leading and lagging strands as template, thereby producing free, double-stranded chromosomal ends (substrates for RecBCD), or they stop behind the first fork leading to eventual breakage and SOS induction.

Accidental replication pauses

In this section, pauses in the progression of replication forks that occur when they encounter nonprogrammed obstacles will be considered accidental. For example, arrest of replication forks and disassembly of the replisome may result from an encounter with DNA lesions, DNA secondary structure, or tightly bound proteins (for review, see Bierne and Michel 1994; Hyrien 1999).

Recently, it was shown that inactivation of helicases involved in replication results in increased recombination, likely due to accidental pausing (Bierne et al. 1997; Saveson and Lovett 1999). Two helicases participate in the replication of the *E. coli* chromosome. DnaB is the main helicase associated with the replisome. Rep is an accessory helicase thought to facilitate the progression of replication forks by dislodging DNA-bound proteins from the path of replication forks (Matson et al. 1994). In the absence of the Rep protein, replication of the chromosome takes twice as long (Lane and Denhardt 1975), suggesting that the replication is arrested frequently. Inactivation of either DnaB or Rep causes the accumulation of DSBs in cells deficient for RecBC (Michel et al. 1997). The enzymes responsible for the DSBs were identified as the RuvABC complex (Seigneur et al. 1998). The RuvAB complex is responsible for branch migration of Holliday junction recombination intermediates and is required for the action of RuvC, the endonuclease that cuts the junctions (for review, see West 1997). Inactivation of RuvAB or RuvC suppresses the occurrence of DSBs in *dnaBts recB* mutants at restrictive temperature, indicating that the RuvABC complex is essential for the appearance of DSBs upon replication arrest in *dnaB* mutants.

The *rep* mutation allowed a more detailed genetic and physical analysis of this process. *rep* mutants require RecBC for growth. This requirement, as well as the occurrence of DSBs, was relieved by mutations inactivating the RuvAB complex (Seigneur et al. 1998). Thus, RuvAB was required for the formation of the double-stranded ends, the targets of RecBC. In contrast, inactivation of the RuvC endonuclease did not restore the viability of *rep recBC* mutants, indicating that double-stranded ends were made in the *rep* strain solely by the action of RuvAB. It was proposed that the RuvAB complex creates a DNA double-stranded end at arrested replication forks by annealing the two nascent strands (Fig. 2A; Seigneur et al. 1998). The resulting structure, akin to a Holliday junction formed during homologous recombination, can be branch migrated by RuvAB and eventually cleaved by RuvC. The RuvAB proteins may act either directly at the fork to create the junction or by stabilizing a structure that forms upon replication arrest. Recombination between the double-stranded tail and the

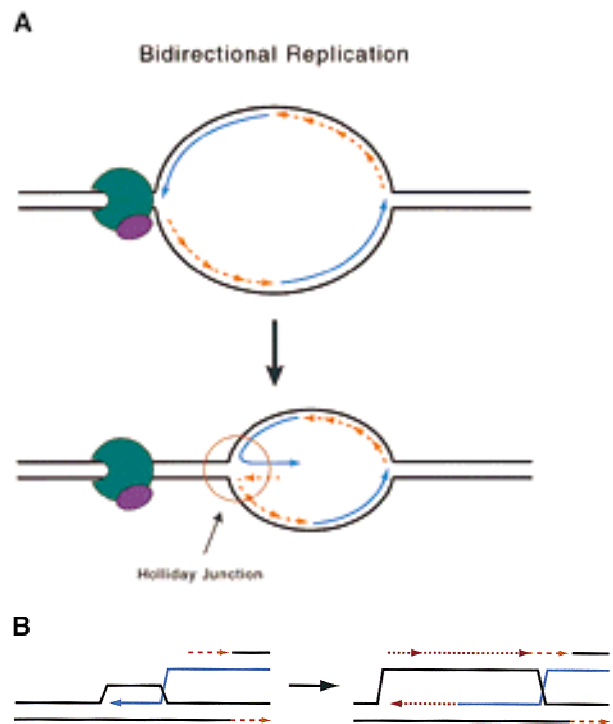


Figure 2. DNA end formation and recombination-initiated restart. (A) A bidirectional replicon is shown confronting a replication fork block (indicated by the green/purple protein complex). Inability to pass the block results in the formation of a free DNA end after annealing of the newly replicated strands and branch migration. The red circle (bottom) indicates the Holliday junction formed during this process. Resolution of the Holliday junction will generate a free end. (B) Break-induced recombination can reform a replication fork. The end generated by cleavage of the Holliday junction invades its homologous sequence, establishing a new replication fork (Malkova et al. 1996; Morrow et al. 1997).

chromosome would facilitate replication restart (Fig. 2B) and may account for the increased recombination between tandemly repeated sequences observed in *dnaB* mutants (Saveson and Lovett 1999). This model provides an explanation for a peculiar property of the *rep* mutation, namely that it is incompatible with *recBC* mutations but compatible with *recA* inactivation. It was proposed that the double-stranded tail, which was extended by branch migration catalyzed by the RuvAB complex, was either recombined with the chromosome or degraded by the exonuclease V action of RecBCD. In *rep recA* double mutants, degradation of this double-stranded tail restores cell viability without homologous recombination.

It is not known whether a similar reaction occurs at specialized arrest sites like the *Ter*-Tus complex. It has been shown that the DnaB helicase may interact specifically with Tus (Sahoo et al. 1995), and this interaction could influence the fate of replication forks blocked at *Ter*. If fork reversal is prevented at *Ter* sites, this would allow direct breakage of the fork, as proposed by Horiuchi and collaborators (Horiuchi and Fujimura 1995), or

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permit the time for a second replication fork to create a double-stranded end or break, as proposed by Hill and collaborators (Sharma and Hill 1995). The increased number of anucleoid cells in *lacZ::Ter recA* cultures suggests the occurrence of extensive DNA degradation, which may be taken as an argument for direct chromosome breakage (Horiuchi and Fujimura 1995; Uzest et al. 1995; Kuzminov and Stahl 1997). However, anucleoid cells were not observed in the *Inv-Ter* strain (Sharma and Hill 1995). A direct measure of DNA breakage in these strains may help answer the question.

Replication restart from pauses

Processing of arrested replication forks into a double-stranded end by fork reversal or direct breakage leads to replication restart from a recombination intermediate. *oriC*-independent replication in SOS-induced *E. coli* cells was shown to depend on homologous recombination and was called recombination-dependent replication (RDR; for review, see Kogoma 1997). RDR requires the assembly on recombination intermediates of a multiprotein complex called the primosome (Marians 1992). Assembly of the primosome to a D loop formed by strand invasion allows the binding of the replicative helicase, DNA polymerase, and primase, the three enzymatic machines that are required for replication initiation. Mutants deficient in primosome assembly, like *priA* mutants, are partly deficient in homologous recombination, indicating that initiation of replication may be required for the completion of some homologous recombination events *in vivo* (Kogoma et al. 1996). More recently, primosome-dependent initiation of replication from a D loop could be reconstituted *in vitro* (Liu et al. 1999). Furthermore, direct evidence for RDR *in vivo* was provided at a molecular level with the use of λ bacteriophages (Kuzminov and Stahl 1999; Motamedi et al. 1999).

The ease of replication initiation from a recombination intermediate may be the reason why some arrested replication forks are transformed into recombination substrates. In addition, recombination may not only provide a way to reinitiate replication at random sites, it may also help to dislodge obstacles. Such obstacles may be DNA-bound proteins or topological barriers. For example, strains that carry an ectopic *Ter* site are dependent on *RecA* for viability. The restoration of viability by homologous recombination suggests that the newly reconstructed replication forks (which would necessarily have been reformed behind the original blockage) are not arrested again. In contrast, in *E. coli rep* mutants, restart is not absolutely dependent on homologous recombination, as the *rep recA* mutants are viable. In *rep recA* double mutants, restart presumably occurs from a Y structure. Similar reactions may occur in wild-type cells, provided that the obstacle that caused the pause has been removed. The poor growth of *priA* mutants (Sandler et al. 1996) suggests that most replication forks do not reach the terminus without arrest and restart by primosome assembly. However, the observation that *recA* mutants

do not grow as poorly as *priA* mutants indicates that homologous recombination is not an essential step in the replication process and that *PriA* may also be needed to restart forks that have not recombined. It should be noted that the D loop used *in vitro* to show primosome assembly-dependent replication (Liu et al. 1999) mimics a Y-arrested fork as well as a recombination intermediate. To what extent the recombination reaction facilitates replication restart, for example, by direct interactions between recombination and replication proteins, remains to be determined.

Replication pauses or barriers in eukaryotes

Although extensive work has been carried out on replication initiation, very little is known about replication termination in eukaryotic chromosomes. Mechanisms comparable to those described for the circular chromosomes of *B. subtilis* and *E. coli* (Hill 1992) have not been observed in eukaryotic chromosomes, which contain multiple origins of replication. Techniques such as fiber autoradiography have provided some insight into replication termination in mammalian cells (Edenberg and Huberman 1975), but, to date, there have been only a few reports of site-specific replication barriers in the genome of organisms other than *E. coli* and *B. subtilis* (Hill 1996). In *Saccharomyces cerevisiae*, replication termination has been observed to occur anywhere within a 4.3-kb region located between two origins on chromosome III by using two-dimensional (2-D) gel electrophoretic replication mapping techniques (Zhu et al. 1992). The absence of a distinct termination site suggests that forks converge at random sites within this region rather than terminate at a specific sequence. However, there are several instances in which replication stalling or termination has been localized to specific areas of the genome.

Nonuniform replication fork movement has been observed in a wide range of eukaryotic species, including yeast (Brewer and Fangman 1988; Deshpande and Newlon 1996), *Tetrahymena* (MacAlpine et al. 1997; Zhang et al. 1997), *Drosophila* (Shinomiya and Ina 1993), frogs (Wiesendanger et al. 1994), plants (Hernandez et al. 1993), mouse (Lopez-Estrano et al. 1998), monkey (Rao et al. 1988), and man (Gahn and Schildkraut 1989; Dhar and Schildkraut 1991). A difference has been established between replication fork pause sites (RFPs) and replication fork barriers (RFBs). RFPs induce a transient stalling of an elongating replication fork (Greenfeder and Newlon 1992; Deshpande and Newlon 1996), whereas RFBs block further progression of an arrested fork (Brewer and Fangman 1988; Little et al. 1993; Wiesendanger et al. 1994). The difference between RFBs and RFPs can be easily uncovered by various 2-D gel electrophoresis analyses, which provide information about the direction of replication fork movement and location of replication origins, termini, and pause sites (for review, see Huberman 1997). The accumulation of replication intermediates with a characteristic configuration (e.g., Y structures, etc.) are displayed as a Y arc on 2-D gels. A paused replication fork appears as an accumulated spot on a nor-

mally smooth replication arc upon 2-D gel analysis, whereas a replication block eliminates the Y arc and only the spot is seen.

RFBs

In all eukaryotic organisms examined, rDNA replication patterns share conserved features. There are major replication initiation sites restricted to the nontranscribed spacer (NTS), and replication forks arrest at the 3' end of the transcription unit at an RFB (Hernandez et al. 1993). The RFB is polar (unidirectional) and efficient in *S. cerevisiae* (Brewer and Fangman 1988; Linskens and Huberman 1988; Brewer et al. 1992; Kobayashi et al. 1992; Lucchini and Sogo 1994) and mouse (Gerber et al. 1997; Lopez-Estrano et al. 1998), polar and apparently somewhat inefficient in *Xenopus* somatic cells and in the yeast *Schizosaccharomyces pombe* (Wiesendanger et al. 1994; Sanchez et al. 1998), and nonpolar (bidirectional) and apparently somewhat inefficient in human cells (Little et al. 1993). Whether completely efficient or somewhat inefficient, in all tested cases the RFB ensures that the majority of replication forks traveling through the transcribed region move in the direction of transcription.

RFB in yeast rDNA (*Fob1* dependent)

The RFB found in the *S. cerevisiae* rDNA is the most well-characterized example in a eukaryotic organism owing to its repetitive nature and the development of 2-D gel technology (Brewer and Fangman 1988; Linskens and Huberman 1988). It was shown to be independent of active transcription, as RFBs are still detected in mutants that do not transcribe the rDNA located in the chromosome. In addition, when RFBs are cloned into a plasmid, unidirectional pausing is still observed, even when transcription is mediated by RNA polymerase II (Brewer et al. 1992; Kobayashi et al. 1992). Interestingly, the RFB was found to overlap with an essential element of the rDNA recombinational hot spot *HOT1* (Keil and Roeder 1984; Voelkel-Meiman et al. 1987). Several years later, the Horiuchi laboratory isolated a gene (*FOB1*) whose product is necessary for both replication fork blocking and recombinational hot spot activity (Kobayashi and Horiuchi 1996), suggesting that homologous recombination at *HOT1* is directly linked to DNA replication fork blocking at RFB. A recent study from the Guarente laboratory has indicated that Fob1 protein is localized to the nucleolus (Defossez et al. 1999); however, it is not yet known whether the Fob1 protein actually interacts directly with DNA at the RFB. The latest studies on Fob1 biology have revealed an involvement of this protein in the regulation of the rDNA copy number that is dependent on a functional RNA polymerase I, as well as an involvement in the aging process (Kobayashi et al. 1998; Defossez et al. 1999; Rothstein and Gangloff 1999). A relationship between extrachromosomal rDNA rings (ERCs) and aging has been established (Sinclair and

Guarente 1997; Sinclair et al. 1997), and there is a correlation between the presence of Fob1 and the production of ERCs (Defossez et al. 1999). This work suggests that a replication fork block can lead to increased levels of recombination, like in bacteria.

RFB in plant rDNA

In the pea, *Pisum sativum*, the strategy used to replicate rDNA is very similar to that used in *S. cerevisiae*. The replication forks moving in the opposite direction to rRNA transcription are stalled at a polar RFB that is also located at the 3' end of the 35S precursor rRNA (Hernandez et al. 1993). The fragment containing the *P. sativum* RFB was subcloned in both orientations into a unidirectionally replicated pUC vector and transformed into *E. coli*. Upon 2D gel electrophoresis analysis, no accumulation of paused replication forks was detected on the plasmids, suggesting that pea *trans*-acting factors and not the DNA structure itself are probably responsible for fork pausing. Recently, imperfect 27-bp tandem repeats have been shown to be involved in the arrest of replication. Additionally, nuclear proteins were found to specifically bind these repeats, supporting the view that a DNA/protein complex is responsible for the polar arrest of replication forks (Lopez-Estrano et al. 1999).

TTF-I-mediated RFB in mammalian rDNA

In human rDNA, a RFB is located at the 3' end of the rRNA transcription unit. However, unlike the situation in yeast or plants, this RFB stalls replication forks in both directions but still limits DNA replication to the same direction as transcription (Little et al. 1993). In addition, termination of replication occurs in this region only in some rDNA repeat units. This region has been studied previously in detail and it contains binding sites for proteins that have a role in the termination of mouse, rat, and human rRNA transcription (Grummt et al. 1985; Kermekchiev and Grummt 1987; Kuhn et al. 1988; Pfliederer et al. 1990). In the mouse, the terminator of transcription is an 18-bp sequence motif called the Sal box, which is repeated 10 times downstream of the 3' end of the pre-rRNA coding region. The Sal box is recognized by the transcription terminator factor TTF-I, which mediates the stop of the elongation reaction of RNA polymerase I (Grummt et al. 1985). The DNA-binding domain residing in the carboxy-terminal portion of TTF-I is highly conserved from mouse to man (Evers and Grummt 1995). This part of the protein also shares strong homologies with the DNA-binding domain of both the proto-oncoprotein c-Myb (Kanei-Ishii et al. 1990) and the yeast transcription factor Reb1 (Morrow et al. 1993). Interestingly, Reb1, the yeast homolog of TTF-I, binds to yeast RNA polymerase I terminator element and mediates transcription termination (Lang and Reeder 1993).

In a recent study using an SV40-based cell-free system, it has been shown that Sal box 2 and its flanking regions

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constitute a polar barrier to replication fork movement. Furthermore, binding of TTF-I to Sal box 2 is a prerequisite for replication fork arrest, and RFB activity occurs in the absence of transcription. A similar result was obtained in a study of replication and transcription termination in the mouse rDNA, where the binding of TTF-I leads to a polar arrest of the replication fork (Lopez-Estrano et al. 1998). The binding of TTF-I may effectively block the progression of the replication fork. Alternatively, the absence of TTF-I may simply permit the transcription machinery to progress beyond the transcription terminator to dislodge some other element farther downstream that specifically blocks replication fork movement.

A viral replication fork block

The Epstein–Barr virus (EBV) latent origin of replication, *oriP*, was identified as a genetic element that confers to small plasmids the ability to replicate autonomously in EBV-transformed cells. In its latent infective cycle, EBV is maintained as a circular double-stranded multicopy episomal plasmid of 172 kb in human B cells. *oriP* is a *cis*-acting element of 1.8 kb necessary to maintain the EBV plasmid in the B cells (Yates et al. 1984). One feature of *oriP* is the presence of 20 copies of a 30-bp tandemly repeated sequence (Reisman et al. 1985). The single viral protein required for *oriP* function, EBNA-1 (Epstein-Barr nuclear antigen-1), binds to a 12-bp consensus palindromic sequence in each repeat. Upon EBNA-1 binding, the bidirectional replication forks encounter a RFB in the region of the EBV repeats, and termination occurs at or in the vicinity of the repeats (Gahn and Schildkraut 1989). Reducing the number of repeats from 20 to 6 has little effect on the strength of the pausing, but EBNA-1 binding is absolutely required (Dhar and Schildkraut 1991). More recently, it was shown that EBNA-1 binding to DNA inhibits the unwinding activity of T antigen (3' to 5') and *dnaB* (5' to 3') helicases in an orientation-independent manner.

RFP

Centromeres in yeast

At about the same time that the RFB in rDNA was shown to be independent of transcription (Brewer et al. 1992; Kobayashi et al. 1992), Newlon and coworkers reported the existence of RFPs at yeast centromeres. Unlike what was observed in rDNA, centromeres are capable of pausing replication forks independently of their direction of progression. However, as in eukaryotic rDNA, this pausing was proposed to depend on the formation of specific protein–DNA structures during S phase (Greenfeder and Newlon 1992). The presence of the centromere protein–DNA complex was shown to result in the pausing of most, if not all, replication forks rather than a complete block of only a subset of progressing forks.

tRNA genes

RFPs have been found to be a general property of tRNA genes in yeast (Deshpande and Newlon 1996). They are polar, stalling replication forks only when they oppose the direction of tRNA transcription. The binding of two transcription factors (TFIIIB and TFIIIC) to their cognate targets is a prerequisite for recruiting RNA polymerase III and transcribing the tRNA gene. The binding of TFIIIC to box B in the tRNA sequence and TFIIIB to a region upstream of the transcription start was shown to be both required but not sufficient for pausing. In addition, binding of RNA polymerase III to the initiation complex on the tRNA gene and possibly transcription *per se* is necessary (Deshpande and Newlon 1996). This suggests that replication and transcription of tRNA genes are concurrent. The polar block indicates that only head-on collisions between the replication and transcription machinery result in significant RFP activity. The polar nature of the block was proposed to result from an accumulation of superhelical density. If the replication and transcription machinery progress toward one another, then positive supercoiling will accumulate and stall each. On the contrary, if the two machines travel in the same direction these effects may cancel out.

Tetrahymena thermophila rDNA minichromosome

During vegetative growth, the rDNA of ciliated protozoan *Tetrahymena thermophila* is replicated exclusively from origins in the 5' NTS. Replication fork pausing takes place in the nucleosome-free regions of the 5' NTS. However, a significant reduction in pausing is observed when the 5' NTS is cloned into a ColE1-derived plasmid introduced into *E. coli*. This observation raised the possibility that either chromatin organization or *Tetrahymena*-specific factors may be required to observe pausing. Mutations in type I elements shown previously to control replication initiation (Larson et al. 1986) also regulate elongation of RFPs. Contrary to what has been observed for barriers or pause sites elsewhere, there is a strong bias in pausing for forks moving in the same direction as transcription (MacAlpine et al. 1997). In *Tetrahymena*, these investigators proposed that pausing coordinates replication and transcription by preventing disruptive rear-end collisions between the two polymerases.

Perspective

Replication fork pausing is conserved from bacteria to humans, and it is likely that this important biological process and the associated activities necessary to manage it are tightly regulated. Very often, pausing is preprogrammed, as seen in the rDNA replicons of many species. The existence of specific replication blocks near highly transcribed rDNA genes suggests that replication and transcription must be temporally and spatially separated to prevent collisions between the replication and transcriptional machinery (Fig. 3; Hill et al. 1988; Brewer

et al. 1992; Deshpande and Newlon 1996). The rRNA cistrons in bacteria are also oriented such that transcription does not collide with either of the two replication forks emanating from the origin (French 1992). In addition, replication is sensitive to DNA secondary structure and pausing often occurs near hairpin sequences. DNA helicase activity may be necessary to relieve such impasses. It is interesting to note that the pausing associated with secondary structure in DNA may also be re-

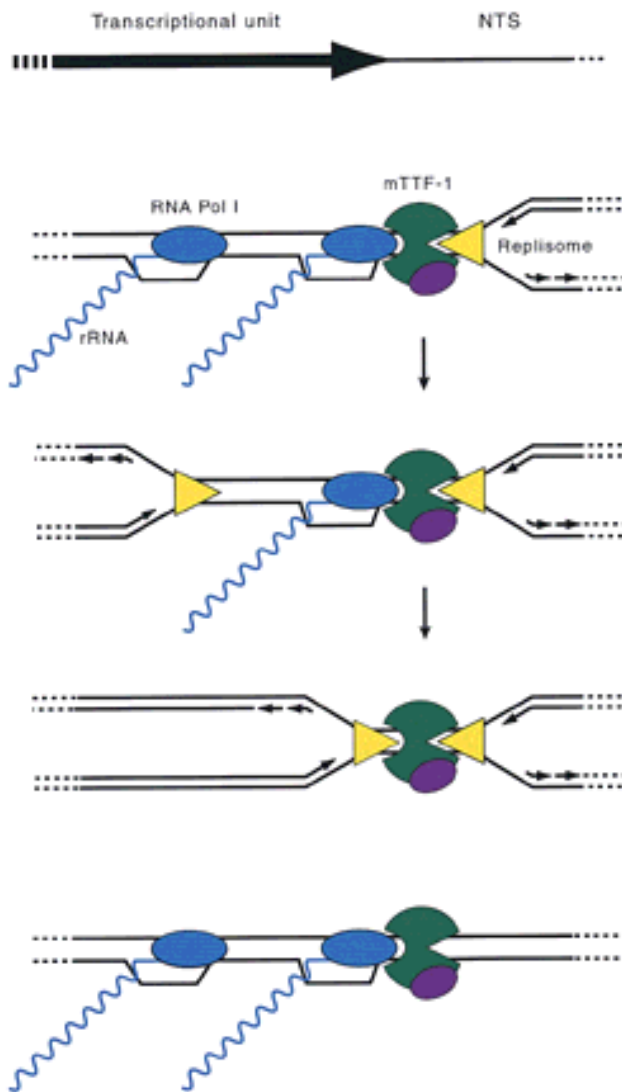


Figure 3. RNA polymerase is depicted as a blue oval. The green ball represents a DNA-binding protein (like mTTF-1) that blocks movement of the replication machinery (yellow triangle) in one direction (from the *right* in this example) and at the same time acts to terminate transcription in the other (from the *left*). The purple oval indicates a hypothetical accessory protein that could help coordinate replicon fusion and transcription. The green colored protein performs both fork blocking and termination functions; however it is not necessary that the activity resides in the same protein. Transcription is terminated before reaching the stalled replication fork, which eventually fuses with the incoming fork on the *left*. Adapted, with permission, from Lopez-Estrano et al. (1998).

lated to transcription. For example, tRNA genes, which like rDNA can easily form secondary structures, also act as natural pause sites in yeast (Deshpande and Newlon 1996). Thus, the complex organization of the replication and transcription machinery has likely evolved to tackle this problem.

Another possible reason for regulated replication pausing is to coordinate replicon fusion. The benefit of tightly regulating this process is to ensure that the cell cycle functions smoothly. Just as initiation of DNA synthesis is regulated so that each replicon is fired only once per cell cycle, the two replication forks must merge prior to chromosome segregation and cell division. This is true for organisms with circular chromosomes as well as for those with linear chromosomes containing multiple origins. Thus, it is likely that regulatory factors control orderly replicon fusion. For example, stalled replication may lead to a broken fork that must reform to continue replication. It is essential to prevent the passage of the adjacent replication fork before restoration. Otherwise, there is the risk of generating local aberrant replication structures, as the broken end will reform a replication fork by invading one of the two strands of the newly replicated chromosome. As a result, these two forks accidentally pass one another, over-replicate the region, and never fuse. Thus, replication blocks or pause sites may serve to direct the binding of the regulatory factors necessary for this orderly fusion and to participate in the signaling of an S-phase checkpoint to prevent over-replication. It is noteworthy that to date, no single mutation that affects replication fork pausing or blocking is lethal, suggesting that this process is so important that backup systems exist to ensure cell survival in the event of a failure.

The faithful replication of DNA requires both helicase and topoisomerase activities. In *E. coli*, mutations in the *rep* and *dnaB* DNA helicases result in increased pausing that must be overcome by recombination (Michel et al. 1997). In yeast, Sgs1 helicase interacts with topoisomerases II and III, which are both likely to have a role in untangling intertwined DNA strands (Gangloff et al. 1994; Watt et al. 1995; Wang 1996). The interactions between these proteins may be part of a control mechanism for replication termination (Gangloff et al. 1999). Loss of function of either *SGS1* or *TOP3* results in increased recombination in the multiple tandem rDNA array (Gangloff et al. 1994). Loss of *SGS1* function also leads to increased rDNA circles. Recently, an inverse correlation between replication pausing and rDNA recombination has been made. Mutations in *FOB1*, which eliminate the RFB, also decrease recombination as measured by marker loss in rDNA as well as by rDNA circle formation. Thus, replication pausing, which may be the result of lesions caused by the absence of enzymatic activities that regulate DNA metabolism or by a replication block per se, can lead to recombination to alleviate the problem.

Work from bacteria has shown that accidentally arrested replication forks recombine to permit replication restart. The current view that recombinogenic DNA

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ends are created is supported by genetic and physical data. In *E. coli*, fork reversal at such accidental replication arrests is one step in a pathway leading to the break. Again, it is apparent that the cell responds to breaks via mechanisms that coordinate the reconstitution of the replication fork. One can take the long-range view that the cell actually takes advantage of the formation of recombinogenic ends at programmed replication arrest sites. For example, in the case of rDNA, it may be beneficial to create breaks specifically in this array, albeit at a low frequency, to stimulate recombination. Thus, rare, but directed, recombinogenic ends are generated to permit the shuffling of DNA sequences within multiple tandem arrays to maintain their sequence homogeneity via gene conversion or to stimulate their loss or gain via direct repeat recombination. This view also provides an alternative explanation for the presence of pause sites near tRNA genes. In eukaryotes, they are members of dispersed multigene families whose sequence homogeneity must be maintained by gene conversion between chromosomes (ectopic recombination). Recombination initiated when pausing takes place at sites near such sequences would have the potential to stimulate ectopic events among members to preserve identity within the family.

In conclusion, the recent breakthroughs in the study of replication pausing in both prokaryotes and eukaryotes foreshadows an exciting era for the unraveling of the components and regulatory mechanisms underlying this highly conserved biological process.

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Replication fork pausing and recombination or "gimme a break"

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