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SURVEY AND SUMMARY

Coordination of DNA synthesis and replicative unwinding by the S-phase checkpoint pathways

Marina N. Nedelcheva-Veleva, Dragomir B. Krastev and Stoyno S. Stoynov*

Institute of Molecular Biology, Bulgarian Academy of Sciences, 21 G. Bonchev Strasse Sofia 1113, Bulgaria

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ABSTRACT

The process of DNA replication includes duplex unwinding, followed immediately by DNA synthesis. In eukaryotes, DNA synthesis is disturbed in damaged DNA regions, in replication slow zones, or as a result of insufficient nucleotide level. This review aims to discuss the mechanisms that coordinate DNA unwinding and synthesis, allowing replication to be completed even in the presence of genomic insults. There is a growing body of evidence which suggests that S-phase checkpoint pathways regulate both replicative unwinding and DNA synthesis, to synchronize the two processes, thus ensuring genome stability.

INTRODUCTION

Fine coordination of several individual processes during DNA replication is required for correct duplication of genetic information and maintenance of genome stability. Recent studies of the physical and functional interaction at replication forks have shed light on the synchronization between DNA synthesis and replicative unwinding.

The MCM heterohexameric helicase complex is essential for DNA unwinding during both the initiation and elongation steps of DNA replication (1). Several regulatory proteins—Cdc45 (2), Mrc1, Tof1, Csm3 (3) and GINS (4) complex interact with MCM. The binding of the DNA polymerization machinery to the origin of DNA replication requires the preloading of MCM complex and its associated factors Cdc45 and GINS, by the ORC/Cdc6/Cdt1 initiation apparatus (5–9), but the exact mechanism by which this is accomplished is still unclear. It was demonstrated that polymerase ε is the first DNA polymerase, which is bound at the origins of DNA replication and is required for polymerase α/primase association with these origins (10,11). The order of polymerase loading is surprising in view of the fact that the DNA synthesis by polymerase ε requires synthesis of a RNA/DNA primer by polymerase α/primase (12).

Several other proteins are necessary for initiation of DNA replication. Dpb11/Sld2 interaction with polymerase ε is involved in binding of polymerases to the origin of replication (10,13). In addition, MCM10 is required to load polymerase α/primase onto the replication forks (14). After the binding of polymerase ε, the four subunit polymerase α/primase complex creates a short RNA/DNA primer as a first step in DNA synthesis of both the leading and lagging strands (15). This primer is then utilized by the PCNA bound polymerase δ for processive elongation of the two strands (15,16). The loading of the sliding clamp PCNA onto the RNA/DNA primer, by the clamp loader protein complex RF-C, catalyses the switch between polymerase α and the polymerase δ (17). In contrast to polymerase δ, polymerase ε is a highly processive polymerase without PCNA (18). Interaction of polymerase ε with GINS complex greatly stimulates its catalytic activity *in vitro* (19). Although the precise role of polymerase ε *in vivo* is still unclear, several findings suggest its catalytic role during DNA replication. Polymerase ε is associated with replication forks during S phase (5,11) and abolishment of its polymerase activity, displays a defect in the elongation step of chromosomal DNA replication (20). However, the catalytic activity of polymerase ε is not required for cell viability, which implies an other important function of this protein (21,22).

It was shown that both polymerase δ (23) and polymerase ε (24) complexes form dimers. Although the precise role of this dimerization remains to be determined, it is thought to play an essential role in the physical connection between the synthesizing machinery of both strands.

STRUCTURAL AND FUNCTIONAL INTERACTION BETWEEN DNA UNWINDING AND POLYMERASE MACHINERY

It is still unclear whether DNA unwinding and polymerase machinery physically interact with each other. The essential methodological problem is that both helicase and DNA polymerases are DNA motor protein complexes therefore, co-purification of their subunits could be either an artefact of their binding to DNA, or a result of specific protein–protein interactions. The absence of co-purification after treatment of DNA with DNAse or ethidium bromide can be interpreted in two ways, either that the polymerase and helicase complexes do not interact with each other, or that their

*To whom correspondence should be addressed. Tel: +359 979 36 89; Fax: +359 2 72 25 077; Email: stoynov@obzor.bio21.bas.bg

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protein–protein interaction requires them to bind to DNA. Despite the difficulties in explaining the co-purification results, several findings suggest that there is a physical interaction between DNA polymerase complexes and replicative unwinding machinery. It was shown that in addition to co-precipitation with MCM helicase (4), GINS proteins form a complex with DNA polymerase epsilon holoenzyme in stoichiometric amounts *in vitro* (19). It has been established that Cdc45 co-precipitates not only with MCM helicase, but also polymerase ε (2). Finally, the MCM complex and the replicative polymerases simultaneously become uncoupled from the inhibited DNA synthesis, in Mrc1 and Tof1 deficient cells (25). However, this raises the question as to whether the protein–protein interactions between DNA polymerase and replicative helicase complexes are sufficient to ensure the coupling of DNA synthesis and replicative unwinding? The flexibility of the ssDNA makes possible the uncoupling of the replicative unwinding and the disturbed DNA synthesis even if the helicase and the polymerase are physically connected (Figure 1C). The estimated persistence

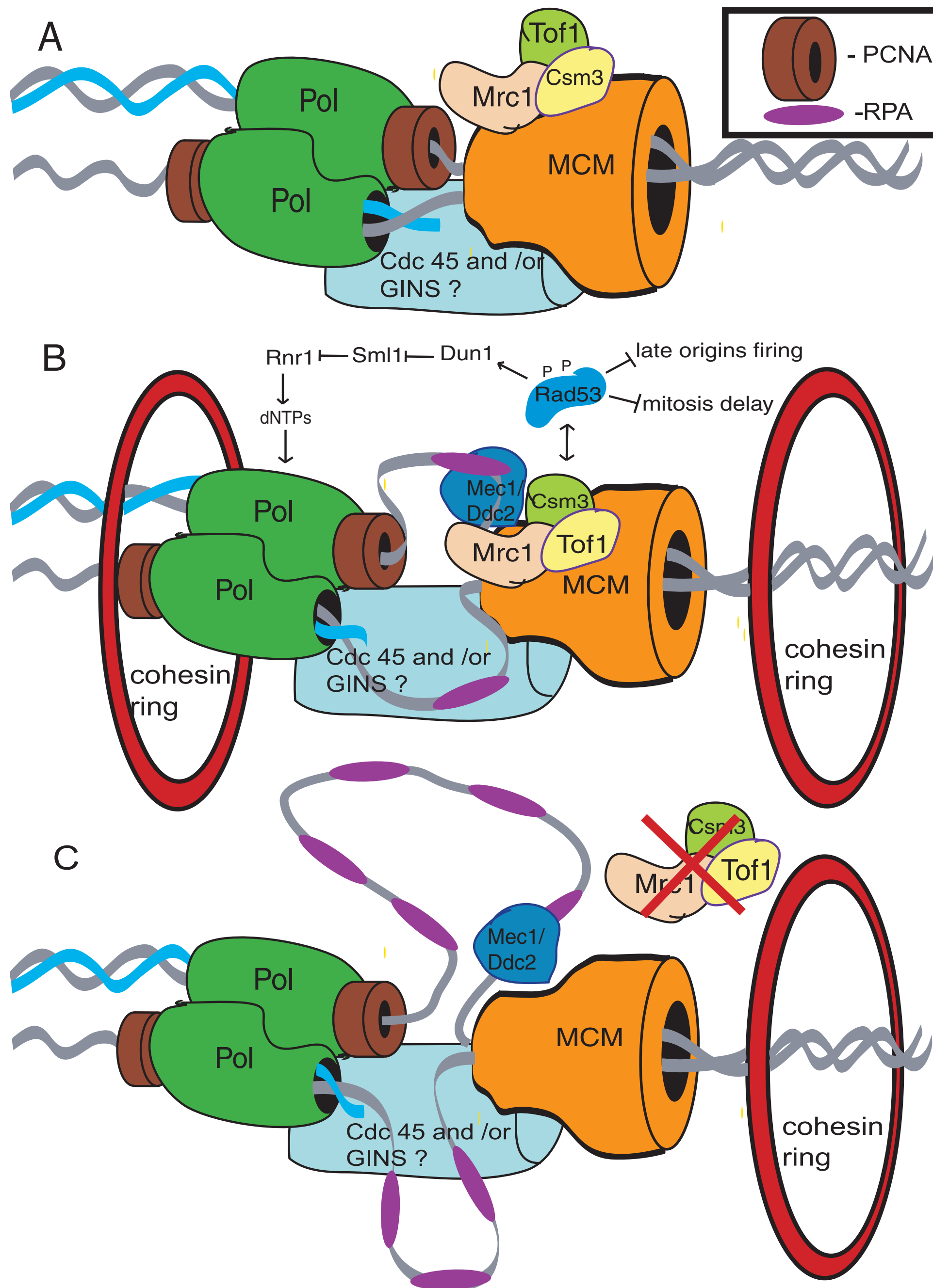

Figure 1. Regulation of DNA synthesis by Mec1-checkpoint pathway. (**A**) Replication fork during unperturbed DNA replication. (**B**) Regulation of DNA synthesis and replicative unwinding when DNA synthesis is disturbed. (**C**) The presence of extensive single stranded loops in *mrc1*, *csm3* and *tof1* mutants impeding the passage through a cohesin ring and the establishment of sister chromatid cohesion.

length of the single stranded DNA, defined as the length at which DNA is capable of bending significantly in two independent directions, ranges from 1.5 to 3 nm (26–28). This means that beyond 5–9 bases ssDNA length of the elastic cost of ssDNA bending is totally negligible.

During normal replication, leading strand DNA synthesis immediately follows replicative unwinding (Figure 1A), which is visualized as a fork branch structure by two-dimensional agarose gel electrophoresis. It was observed that single-stranded DNA (ssDNA) at replication forks is ~200 bp long, most probably as a result of its engagement with replisome (29). When replication forks stall at replication slow zones, damaged DNA regions, or are inhibited by hydroxyurea (HU), yeast cells activate the Mec1-dependent checkpoint pathway (30). This process stabilizes the replication complex to facilitate the re-establishment of fork progression after the stress has been removed (31,32). The best known difference between stalled and normal replication forks is an increase in the single-stranded gap. When the movement of the fork is arrested by HU, the ssDNA region becomes approximately 400 nucleotides long (29). When DNA synthesis is arrested by HU in checkpoint deficient yeast strains, much longer ssDNA regions accumulate (29). All these findings suggest that the Mec1-checkpoint pathway could cause DNA synthesis and replicative unwinding to couple in budding yeast.

MEC1-REPLICATION CHECKPOINT PATHWAY

Mec1 is a member of the phosphoinositide-3-kinases (PIKKs) and is an essential component of the replication checkpoint pathway (30,33–35) in budding yeast. Mec1, in complex with Ddc2 (36,37), recognizes ssDNA coated by replication protein A (RPA) (38,39). The recruitment of Mec1-Ddc2 to the replication intermediates, containing ssDNA regions, is required to activate replication and DNA damage checkpoints.

Other essential players in these checkpoints are the Mrc1, Tof1 and Csm3 budding yeast proteins (40–44) which form a three-protein complex (3). These three proteins co-localize with both normal and stalled replication forks (25,45), all of which are required to slow down DNA replication, for full activation of Rad53 either in response to reduced levels of dNTPs, or to DNA damage (42,45–47). The phosphorylation and activation of Rad53 in a Mec1-dependent manner, leads to the stabilization of stalled replication forks, inhibition of late origin firing and a delay in S/M-phase cell cycle transition (48). The activation of Rad53, in response to inhibition of DNA synthesis by HU, requires Mrc1 phosphorylation by Mec1 (45,49).

MEC1-CHECKPOINT PATHWAY REGULATION OF DNA UNWINDING

Several recent findings suggest that Tof1, Csm3 and Mrc1 are required to regulate replicative unwinding by the Mec1-checkpoint pathway.

When synthesis is inhibited by HU, deletions of Mrc1, or Tof1, lead to the uncoupling of Cdc45, MCM complex and replicative polymerases from DNA synthesis (25). Tof1, Csm3 and Mrc1 checkpoint proteins interact with different subunits of the MCM helicase complex (3). In addition, the synthetic lethality of the double mutants, carrying deletions in *tof1*, *csm3* or *mrc1* and temperature-sensitive mutations in one of the polymerase α/primase subunit genes, suggests that the Mrc1/Tof1/Csm3 checkpoint complex, can prevent the lethality of cells in which DNA unwinding can proceed without synthesis (3). Recently, it was found that the metazoan Mec1 homolog, the ATR kinase, directly phosphorylates the subunits of the MCM helicase complex. It was observed that the ATR phosphorylates the Mcm2 subunit in human and *Xenopus,* in response to DNA damage and stalled replication forks (50,51). In addition, ATR-interacting protein ATRIP, homologous to budding yeast Ddc2, directly interacts with the Mcm7 (50). ATM, the other metazoan phosphoinositide-3-kinase, required for the activation of the replication and DNA damage checkpoint, phosphorylates Mcm3, as a result of ionizing irradiation (50). Finally, Mcm4 is extensively phosphorylated in HeLa cells when they are incubated in the presence of inhibitors of DNA synthesis or are exposed to UV irradiation (52). Apart from MCM complex, ATR/ATRIP directly interacts with the Timeless protein, human homolog of Tof1 (53). How the interactions between ATR/ATRIP, MCM and Mrc1/Tof1/Csm3 complexes and ATR/ATM phosphorylations of Mrc1 and MCM contribute to the stalling of the replication fork is still unclear. It was shown that the deletion of *mrc1* or *tof1* is sufficient for the uncoupling of Cdc45 and the MCM complex from DNA synthesis during HU arrest (25). In the double *mec1*/*tel1* deletion mutant the co-factor of MCM helicase Cdc45 almost disappears from DNA during HU arrest, indicating a more profound effect of this mutant in the stalling of replication forks (25).

MODELS FOR REGULATION OF DNA UNWINDING BY THE MEC-DEPENDANT CHECKPOINT MECHANISM

The above findings suggest possible mechanisms for the regulation of replicative unwinding when synthesis is disturbed. Pausing of DNA synthesis in response to a decrease in nucleotide levels, or DNA damage (thymine dimers, photo adducts, alkylated bases, etc.), leads to an accumulation of ssDNA regions as a result of the uncoupling of DNA unwinding from synthesis (Figure 1B). RPA mediates Mec1-Ddc2 binding to the single-stranded regions (39), and allows Mec1 to interact with and phosphorylate MCM complex and Mrc1/Tof1/Csm3. These interactions could change the binding of Mrc1/Tof1/Csm3 to MCM complex and inhibit the helicase activity of the latter. The ability of Mrc1 of *Schizosaccharomyces pombe* and claspin (the human homolog of Mrc1) to bind DNA *in vitro* could play an important role for inhibition of the helicase movement (54,55).

In eukaryotes, replication forks also stall at natural site-specific sequences, called 'replication fork barriers'. These sites cause polar fork arrest, which is required for various cellular events, including mating-type switching in *S.pombe* (56) and extrachromosomal ribosomal circle DNA formation in eukaryotes (57,58).

It is interesting that Swi1 and Swi3 (the *S.pombe*'s homologs of Tof1 and Csm3), but not Mrc1, are required for stalling of replication forks at five out of six yeast fission

replication fork barriers (58,59). Mrc1 is also not required for the stalling of replication forks at the replication fork barriers in budding yeast (60). In this case, Mrc1 could be replaced in Tof1/Csm3 complex by another protein, such as Fob 1 in budding yeast (61) and Reb1 in *S.pombe* (62). These proteins bind to the replication fork barrier at the ribosomal locus, which is required for replication fork stalling at this sequence.

Apart from the uncoupling of Cdc45, MCM and DNA polymerases from disturbed DNA synthesis, deletion mutants of *MRC1* or *TOF1* express two additional phenotypes. *Mrc1*, *csm3* and *tof1* mutants exhibit mild defects in sister chromatid cohesion (63–65) and slow down the unperturbed DNA replication in Tof1 and Mrc1 deletion cells (45,66,67). It is still unknown, whether these phenotypes are due to the separate function of Tof1/Csm3/Mrc1 or are a consequence of the uncoupling of DNA synthesis and replicative unwinding. The relationship between the processes of replication and sister chromatid cohesion provides possible explanation for these two phenotypes. If the helicase and the polymerase complexes physically interact with each other, then, uncoupling of DNA synthesis and unwinding in Tof1/Csm3/Mrc1 deficient cells will generate single-stranded loops (Figure 1C). Generation of such loops would hinder sister chromatid cohesion. According to one of the proposed models, supported by Nasmyth's results, cohesion is established by the cohesin rings (68), which encircle the two sister chromatids (69). These rings are loaded onto DNA during S phase. The passage of the replication fork through a cohesion ring is supposed to ensure the cohesion of the two newly synthesized DNA strands. In our opinion the presence of extensive single stranded loops, in *mrc1*, *csm3* and *tof1* mutants, would impede the passage through a cohesin ring and the establishment of sister chromatid cohesion (Figure 1C). Difficulties in passing through these loops could also explain slower DNA replication in Tof1 and Mrc1 deletion cells (45,66,67).

REGULATION OF DNA SYNTHESIS BY THE MEC1-CHECKPOINT PATHWAY

The Mec1-checkpoint pathway not only regulates the activity of the MCM helicase, but also controls DNA synthesis. It has been shown that the Mec1/Rad53-pathway increases the level of nucleotides during S-phase and in response to DNA damage (70). Cells deficient in Mec1 activity exhibit a 25% reduction in the level of dNTPs. The ribonucleotide reductase (RNR) catalyzes the synthesis of dNDPs from NDPs (71). In response to DNA damage, Mec1 activates the Dun1 kinase, that leads to the transcriptional induction of RNR (72,73) and the inhibition of its repressor Sml1 (74), thus increasing dNTP level. It appears that higher levels of dNTPs help the polymerase machinery to replicate replication slow zones and damaged DNA regions faster and more effectively.

All these results suggest that when synthesis is delayed by DNA lesions, the S phase checkpoint pathways both stall replicative unwinding and increase level of DNA synthesis, synchronizing the two processes. Synchronization of DNA synthesis and unwinding by the Mec1 pathway could explain the Mec1 mutant phenomena. Mec1-deficient cells die as a result of generation of double-stranded breaks in the replication slow zones (75). This lethality is suppressed by additional deletion of *sml1,* which increases nucleotide level. Both, the slowing of DNA synthesis and the uncoupling of the MCM helicase in Mec1-deficient cells, lead to generation of ssDNA at the replication slow zones. The instability of ssDNA leads to generation of double-stranded breaks and cell death.

UNWINDING OF DNA, CONTAINING SINGLE-STRANDED REGIONS, ACCORDING TO THE VARIOUS MECHANISMS FOR HELICASE ACTION

What happens when the replicative helicase unwinds DNA containing a single-stranded gap or nick? Such sites are frequently generated during both nucleotide and base excision repair or ionizing radiation. The continued replication of such a DNA template would lead to the generation of a double-stranded break in one of the newly synthesized DNA molecules, which is the worst possible scenario for the cell. It is interesting to know how the replicative DNA helicases unwind DNA containing a single-stranded break, according to the different suggested models for helicase action.

In the 'wedge model', also referred as to the 'steric-exclusion model' (Figure 2A), one of the separated strands tightly binds to the central helicase channel. The helicase moves unidirectionally along the strand bound to its central channel, and the movement provides sufficient force to enable the helicase to destabilize the base pairs at the DNA duplex junction (76). When a helicase, functioning in this way, encounters a single-stranded break, the break is transformed into a double-stranded break, in one of the daughter DNA molecules (Figure 2B and C). When the ssDNA break has occurred on the strand where the helicase is moving, the helicase is unloaded from DNA (Figure 2B).

Recently a variant of the wedge model was proposed, that was referred to as the 'ploughshare model' (77). It was postulated that the MCM complex translocates along duplex DNA and that strand separation is achieved by a protein that sterically separates the two strands after they exit from the helicase (Figure 2D). When a 'ploughshare' type helicase encounters a single-stranded break, this break will be transformed into a double-stranded break in one of the daughter DNA molecules; however, the helicase will not be unloaded from DNA.

In the 'torsional model', the two strands are tightly bound to the central helicase channel (76). By rotating the two strands with respect to each other, the helicase generates negative superhelical stress that destabilizes the duplex DNA (Figure 3A). The passage of helicase through a single-stranded break would lead to relaxation of the negative superhelical stress behind the ssDNA region. Therefore, the DNA helix cannot be unwound (Figure 3B), and the replication fork would stall without generation of a double-stranded break in the daughter DNA and unloading of the MCM helicase. The maintenance of the MCM helicase on DNA is important since, until now, the re-loading mechanism of MCM onto DNA during S-phase has not been detected.

The torsional model of DNA unwinding requires superhelical tension to be preserved in the region, between the

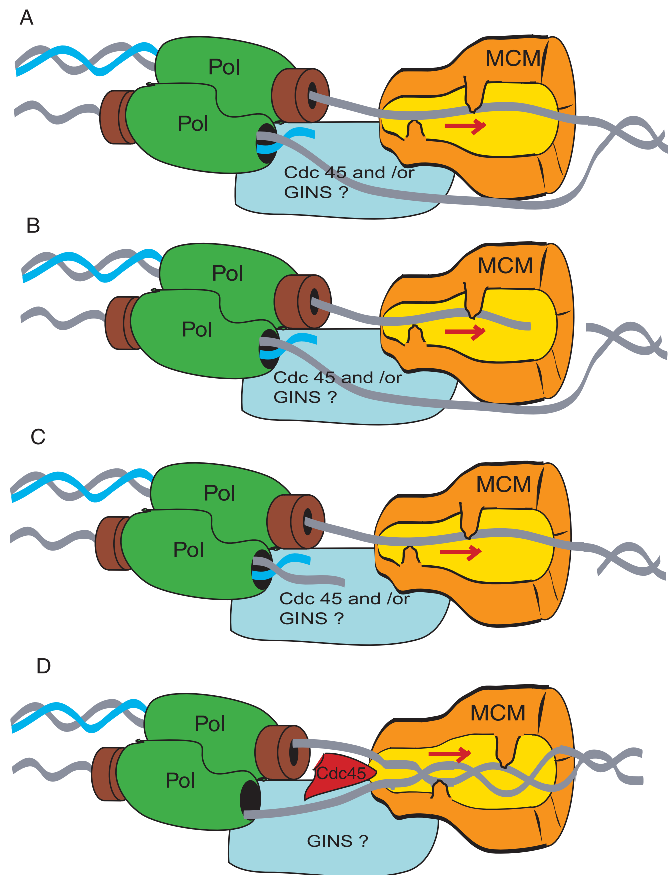

Figure 2. Unwinding of DNA containing ssDNA break according to the 'wedge' and 'ploughshare' model. (**A**) DNA unwinding, according to the 'wedge' model. (**B**) Unwinding of DNA, containing ssDNA break at strand where the helicase is moving, according to the 'wedge' model. (**C**) Unwinding of DNA, containing single-stranded break at the opposite DNA strand according to the 'wedge' model. (**D**) Mechanism for DNA unwinding based on the 'ploughshare' model.

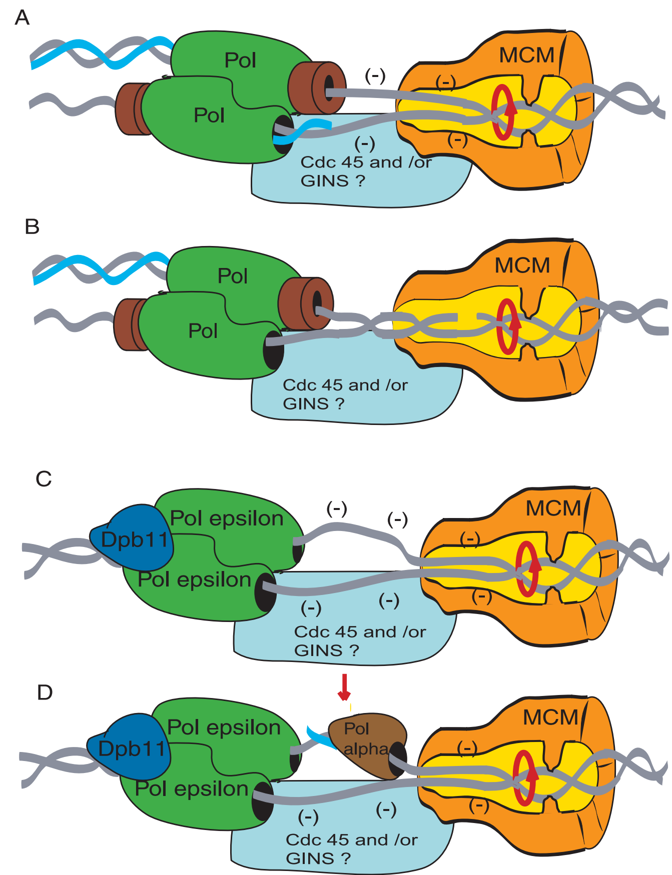

Figure 3. Unwinding of DNA containing ssDNA break according to the 'torsional' model. (**A**) Mechanism for DNA unwinding based on the 'torsional' model. (**B**) DNA unwinding when there is an ssDNA break, according to the 'torsional' model. (**C** and **D**) Origin DNA unwinding and loading of polymerase α/primase according to the 'torsional' model.

replicative helicase and the polymerase complexes. The dimerization of the leading and lagging strand polymerase ε (or polymerase δ) complex and its binding to the MCM via GINS (4,19) and Cdc45 (2,4) can ensure a topologically independent DNA region between them, for the preservation of negative supercoiling (Figure 3A). Several findings indirectly support such a structural role of the polymerase ε. The C-terminal part of the catalytic subunit of polymerase ε, which is responsible for its dimerization but not for polymerase activity, is essential for cell viability (21,22). Polymerase ε loading at the origins of DNA replication is required for polymerase α/primase association with these fragments (10,11), which suggests its role in the unwinding at the origin of DNA, to ensure a template for synthesis of RNA/DNA primer (Figure 3C and D).

In a third model, named the 'rotary pump model', two MCM complexes, located at a distance from each other, pump DNA in opposite directions (78). According to this model, the generated superhelical stress would unwind DNA between the two MCM hexamers (Figure 4A). The MCM helicase has to be immobilized, to induce enough torsional stress to unwind the DNA. Introduction of a single-stranded break in that region could lead to a relaxation of the negative superhelical stress, thus preventing DNA unwinding and the generation of a double-stranded break (Figure 4B). Recently, it was shown that the helicase complexes in vertebrates are detected at the replication fork (79), which is not easily explained by rotary pump unwinding at a distance. In a variant of the 'rotary pump model', two hexamer helicases are not located on distance but are bound to each other to pump DNA in opposite directions (80). This model is supported by the double hexameric structure of SV40 T-antigen replicative helicase (81,82). Archaeal MCM complex also forms a double hexamer (83,84). The binding of the two hexamers, could induce sufficient torsional stress for DNA unwinding (Figure 4C and D). In contrast to SV40, T-antigen and archaeal MCM complex, recent experiments suggest that metazoan MCM complex is a monomer on the replication fork (4). It is interesting that the capability of SV40 T-antigen to maintain torsional tension by its double hexamer organization makes the structural role of polymerase ε unnecessary. In fact, polymerase ε is not required for SV40 T-antigen dependent DNA replication (85,86).

Currently, there are no efficient experimental systems for the investigation of the unwinding of DNA containing a single stranded break. The inhibition of topoisomerase I leads to an accumulation of single-stranded breaks in DNA (87).

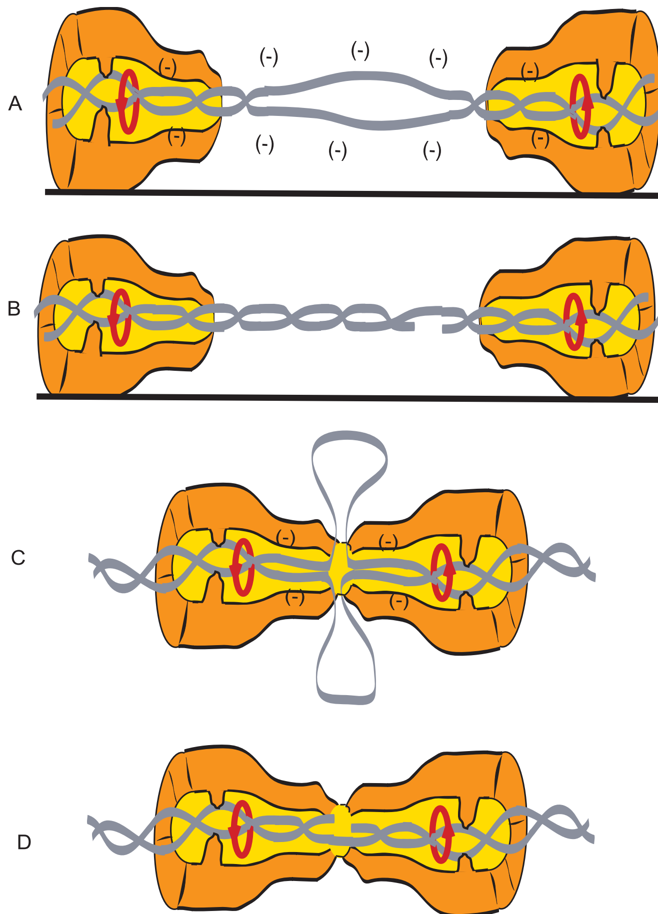

Figure 4. Unwinding of DNA containing ssDNA break according to the 'rotary pump' model. (**A**). DNA unwinding, according to the 'rotary pump' model. (**B**) Unwinding of DNA, containing ssDNA break based on the 'rotary pump' model. (**C**) Mechanism for DNA unwinding based on the 'SV40' model. (**D**) Unwinding of DNA, containing single-stranded DNA break based on the 'SV40' model.

However, in this case the end of the single-stranded break becomes covalently linked to the Topoisomerase I (87) molecule. This makes it impossible to determine whether the experimental results are a consequence of the single-stranded break or of the covalently bound protein. A promising model for ascertaining the true mechanism of DNA unwinding, is the mating-type switching in *S.pombe*. Mating-type switching occurs when the *mat1* allele is replaced with the opposite mating-type allele by recombination with one of the two transcriptionally silent donor cassettes *mat2P* or *mat3M* (88,89). Mating-type switching is a process that requires two rounds of DNA replication. During the first round, a stable single-stranded imprint is made at the *mat1* locus that remains in the DNA throughout the next cell cycle. During the following round of DNA replication, the replisome encounters the imprint, which leads to recombination and mating-type switching. Recent studies suggest that the imprint at *mat1* is either a ssDNA break (90,91) or an alkali–labile DNA modification (92–94). Using the mating-type switching for investigation of the DNA unwinding mechanism requires clarification as to whether the imprint is a single stranded DNA break.

REPLICATIVE DNA UNWINDING IN *XENOPUS* EMBRYONIC CELLS

Whether the ATR- (Mec1-) checkpoint pathway coordinates the replicative unwinding and DNA synthesis in all eukaryotic cells is still unclear. It is known that SV40 T-antigen unwinds DNA *in vivo* when DNA synthesis is inhibited (95). This could be explained by the fact that SV40 virus T-antigen helicase is not under the control of the host Mec-1 checkpoint pathway. This is not the case with *Xenopus* and sea urchin embryos. In their cells, few if, any replication forks were observed (96,97). Instead, unbranched DNA that was suggested to be single-stranded, was abundant during the S-phase (96,97). This phenomenon is difficult to explain in terms of coupling of replicative unwinding and DNA synthesis. In the *Xenopus* egg extract, plasmid DNA was completely unwound when DNA synthesis was inhibited either by aphidicolin (98) and *cis*-platinum treatment or by UV irradiation (99); however, the replicative checkpoint in this extract seems to be at least partially active. Claspin and Chk1 proteins are phosphorylated by ATR, when DNA synthesis is inhibited (47,100). The phosphorylation of Chk1 requires DNA to be unwound to allow ATR-ATRIR to bind ssDNA via the RPA proteins (99). In addition, as indicated above, ATR directly phosphorylates the MCM helicase as a result of checkpoint activation (50,51). Why the ATR phosphorylation of MCM, claspin and Chk1 delays mitosis, but does not slow down the replicative unwinding in *Xenopus* embryos is still unclear. One possible reason could be in Tof1 and Csm3 homologs which have not been identified at the replication forks in the *Xenopus* egg extract.

Finally, we can conclude that coordination of unwinding and DNA synthesis by the S-phase checkpoint pathway is required for correct DNA replication and the maintenance of genome stability. Future investigations will reveal in detail the intimate mechanisms that lead to regulation of the MCM replication fork stalling activity.

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