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Resolution of head-on collisions between the transcription machinery and bacteriophage Φ29 DNA polymerase is dependent on RNA polymerase translocation

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The outcome of collisions between *Bacillus subtilis* phage Φ29 DNA polymerase and oppositely oriented transcription complexes has been studied *in vitro*. We found that the replication fork was unable to go past a transcription ternary complex stalled head-on. However, head-on collisions did not lead to a deadlock. Both DNA and RNA polymerase remained bound to the template and, when the halted transcription complex was allowed to move, the replication machinery resumed normal elongation. These results suggested that a replication fork that encounters an RNA polymerase head-on whose movement is not impeded would bypass the transcription machinery. Our results for head-on collisions between concurrently moving replication and transcription complexes are indeed consistent with the existence of a resolving mechanism. The ability of Φ29 DNA polymerase to resolve head-on collisions with itself during symmetrical replication of Φ29 DNA *in vivo* is likely to be related to its ability to pass a head-on oriented RNA polymerase.

Keywords: ϕ29 DNA replication/replication fork arrest/replication–transcription collisions

Introduction

Propagation of a replication fork can be altered by several factors such as DNA structure (Hacker and Alberts, 1994; Usdin and Woodford, 1995; Krasilnikov *et al*., 1997), the binding of specific proteins to their recognition sequences (see Hill, 1992; Baker, 1995; Murthy *et al*., 1998) and transcription (French, 1992; Liu and Alberts, 1995; Deshpande and Newlon, 1996; Elías-Arnanz and Salas, 1997; Krasilnikova *et al*., 1998). Transcription-dependent stalling of replication forks appears to lie in the occurrence of collisions between RNA polymerases (RNAP) and DNA polymerases (DNAP). Unless transcription and replication are spatially or temporally separated, occasional collisions between the two enzymatic complexes involved seem unavoidable. In studies of *Escherichia coli* rRNA genes (French, 1992) and *Saccharomyces cerevisiae* tRNA genes (Deshpande and Newlon, 1996), transcription interfered *in vivo* with replication fork progression only when occurring in opposite directions. The apparent evolutionary selection for codirectionality of replication and transcription in *E.coli*, several *E.coli* phages and *Bacillus subtilis* (Brewer, 1988; Zeigler and Dean, 1990) also points to head-on collisions being more difficult to resolve than codirectional collisions. Direct *in vitro* assessment of the impact of codirectional and head-on collisions is possible with the defined replication system of the *B.subtilis* bacteriophage ϕ29. Replication of the linear genome of ϕ29 proceeds efficiently *in vitro* in the presence of the phage-encoded DNAP and terminal protein (TP). DNAP as a heterodimer with TP catalyses the initiation of DNA replication at each genomic end by the addition of the first dAMP to a specific hydroxyl group in the TP (reviewed in Blanco and Salas, 1996). After this protein-primed initiation step, TP remains linked to the DNA ends, and DNAP processively replicates one of the DNA strands in the absence of any accessory factors (Blanco and Salas, 1996). Given that replication can start at either genomic end, the replication fork can collide in a codirectional or a head-on manner at any ϕ29 transcription unit. Thus, co-orientation of replication and transcription has not been evolutionarily selected in ϕ29, making it an ideal system for evaluating the consequences of DNAP and RNAP encountering each other. We showed previously that ϕ29 DNAP lacks a mechanism to resolve codirectional collisions with the transcription machinery (Elías-Arnanz and Salas, 1997). Upon collision with a stalled ternary complex, replication fork advance was completely blocked; the DNAP remained associated with the template and was able to resume elongation once the transcription complex was allowed to translocate. In experiments with concurrent replication and transcription, an important decrease in the rate of replication fork progression was observed. Hence, we proposed that a replicating ϕ29 DNAP that codirectionally encounters a more slowly moving RNAP would be forced to slow down, and will translocate behind the RNAP until the latter leaves the template. Our results with the ϕ29 system were of particular relevance because they differed from those of the only other *in vitro* system where this problem was addressed (Liu *et al*., 1993, 1994). In these latter studies of codirectional collisions between the T4 replication apparatus and *E.coli* σ70 RNAP, the DNA replication fork managed to bypass both stalled and moving transcription complexes, without perturbing transcriptional elongation. However, this mechanism to pass codirectional obstructions may not apply generally, as was shown by our results in the ϕ29 system.

In this study, we have examined the outcome of head-on collisions between replication and transcription in ϕ29. As in the case of codirectional collisions, we find that stalled transcription complexes are very efficient barriers for replication forks advancing head-on. Again, our results contrast with those obtained with the T4 replication

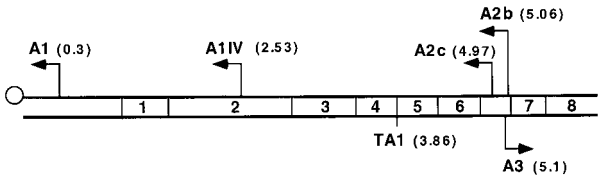

Fig. 1. Transcriptional units in the 6145 bp *Cla*I B DNA fragment. The direction of transcription from each promoter is indicated by arrowheads. Numbers in parentheses represent the positions (kb) at which transcription initiates from each promoter. Numbers 1–8 refer to the genes included in this fragment. The location of the only transcriptional terminator in this DNA fragment (TA1) is also indicated. The TP is depicted attached to the replication origin (the left 5′ end).

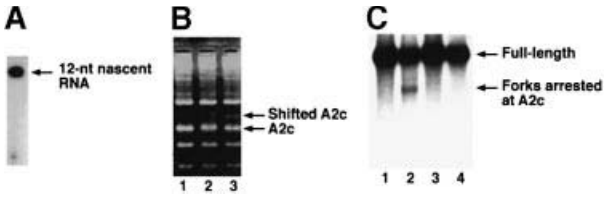

Fig. 2. Collisions between the replication fork and a transcription ternary complex halted at the A2c promoter. (**A**) A transcription ternary complex bearing a 12 nt nascent RNA is formed when the *Cla*I B DNA fragment is incubated with RNAP, [α-^{32}P]ATP, GTP and UTP. The transcription reactions were spun through gel filtration columns prior to analysis by 8 M urea–20% polyacrylamide gel electrophoresis. (**B**) Gel shift assay to estimate occupancy of the A2c promoter by ternary complexes. The positions at which the DNA fragment containing the A2c promoter runs with and without transcription ternary complexes are indicated. Occupancy was determined by densitometric scanning of the retarded band. (**C**) Replication fork arrest due to collision with the ternary complex at A2c. In lane 4, rifampicin was added to the reaction prior to RNAP to inhibit the formation of ternary complexes.

system, where the replication fork was able to pass an oppositely oriented stalled transcription complex (Liu and Alberts, 1995). Interestingly, when the ternary transcription complex was allowed to move, ϕ29 DNAP could also resume elongation. This suggests that a resolution mechanism for head-on collisions in ϕ29 exists only when both complexes are actively moving in opposite directions.

Results

Head-on collisions with a stalled ternary complex interfere with replication fork advance

Our first approach to determine the effect of head-on collisions with the transcription machinery on replication fork progression was to analyse the outcome of such collisions with a stalled transcription complex. Treatment of ϕ29 TP–DNA with *Cla*I generates two fragments (A and B) that can be purified from each other by sucrose gradient sedimentation as replication-competent DNA fragments (see Elías-Arnanz and Salas, 1997). Transcription from all of the promoters in the *Cla*I B fragment, with the exception of the late A3 promoter (whose transcription is negligible in the absence of the ϕ29 regulatory protein p4), proceeds in the direction opposite to that of *Cla*I B DNA replication (see Figure 1). Thus, replication forks advancing from the only TP-linked ϕ29 DNA end in the *Cla*I B fragment could undergo head-on collisions with transcription ternary complexes stalled at any of these promoters. Template DNA bearing stable ternary complexes at the A2c promoter can be obtained in the presence of ATP, GTP and UTP; lack of CTP would make the RNAP halt 12 nt downstream of the initiation site at A2c. With this subset of nucleotides, very short transcripts (1 nt at A1 and A1IV, and 5 nt at A2b) would form at the remaining promoters. Figure 2A shows that a stable 12 nt nascent RNA (as judged by its ability to survive gel filtration) is indeed the major transcript formed when the *Cla*I B fragment is incubated with RNAP in the presence of ATP, GTP and UTP, as expected for transcription halting at A2c. The formation of a stable ternary complex at A2c was further confirmed by gel retardation analysis. The level of occupancy of the A2c promoter by ternary complexes can be followed after cleavage of the *Cla*I B DNA with *Hin*dIII, and separation of the resulting fragments by agarose gel electrophoresis (see Materials and methods). In reactions containing RNAP but lacking NTPs, no change in mobility of the 759 bp DNA fragment containing the A2c promoter was observed (Figure 2B, lane 2). In contrast, a shift in the mobility of ~18% of the 759 bp DNA fragment was observed when RNAP as well as ATP, GTP and UTP were present, suggesting that the A2c promoter is occupied by ternary complexes in ~18% of the template DNA molecules (Figure 2B, lane 3). Figure 2C (lanes 1 and 2) shows the products of replicating mock-treated DNA (*Cla*I B DNA subjected to the same incubation treatment as in lane 2, but in the absence of RNAP) and *Cla*I B DNA bearing the ternary complex at A2c in ~18% of the template molecules. The expected 6145 bp product was obtained in replication reactions containing mock-treated *Cla*I B DNA as template. When the DNA molecules bearing the ternary complex at A2c were replicated, an additional band mapping to a position where collisions with the ternary complex at A2c would be expected to occur (at ~4900 bp) was observed. This band disappeared when *Cla*I B DNA pre-incubated with only RNAP (no NTPs) was used as the template, or when ternary complex formation was prevented by the addition of rifampicin (an inhibitor of transcription initiation) to the reaction prior to RNAP (Figure 2C, lanes 3 and 4). Altogether, these observations strongly suggest that the shorter product originates from replication fork arrest due to collision with a transcription complex halted at A2c. Replication fork stalling was estimated at ~20% by densitometric scanning. This amount matched an equivalent decrease in the yield of full-length DNA and closely resembles the estimated values for A2c occupancy. We can therefore conclude that the ternary transcription complex halted at A2c efficiently blocks progression of replication forks moving head-on.

The consequences of head-on collisions with transcription complexes halted at other promoters in the *Cla*I B DNA fragment could not be studied due to failure to obtain stable ternary complexes at any of these promoters using limited subsets of nucleotides. Thus, to verify whether our conclusions are applicable to other ϕ29 promoters, we determined the outcome of head-on collisions with ternary complexes formed at a promoter located outside the *Cla*I B DNA fragment: the C1 promoter (see Figure 3A). We have shown previously that RNAP starting transcription at C1 in the presence of ATP, CTP and GTP will halt and form a stable ternary complex with a 14 nt

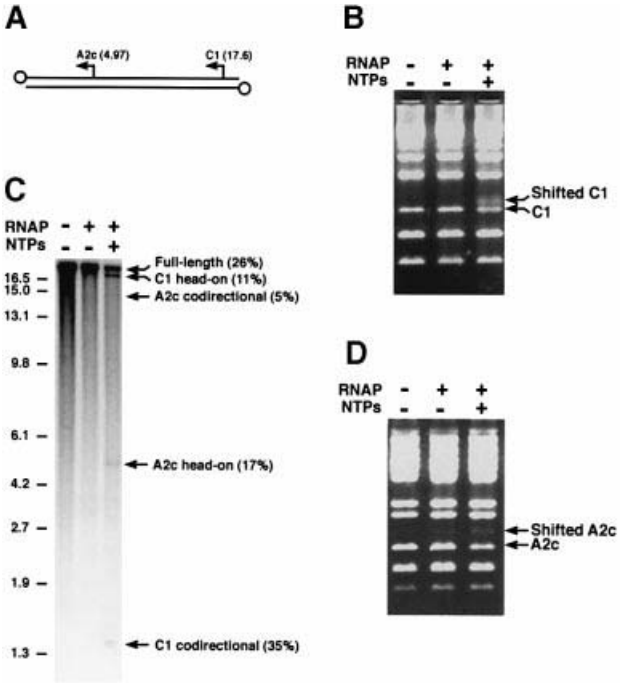

Fig. 3. Replication fork arrest due to head-on collisions with other ternary complexes. (**A**) Schematic representation of the 19 285 bp ϕ29 linear genome showing the positions (kb, from the left end) of the A2c and C1 promoters. The TP is shown covalently linked to both replication origins. (**B**) Occupancy of the C1 promoter by ternary complexes. Whole TP–DNA bearing ternary complexes formed in the presence of RNAP, ATP, CTP and GTP were cut with *Eco*RI and *Ssp*I, as described in Materials and methods. The positions at which the DNA fragment containing the C1 promoter runs when free of ternary complexes and when occupied are indicated. (**C**) Replication of whole TP–DNA bearing ternary complexes at C1 and A2c. Band positions corresponding to full-length DNA and to replication arrest are indicated by arrows on the right. Numbers in parentheses indicate the percentage that each band represents, estimated as described in Materials and methods. On the left, the positions at which specific size markers (kb) run are indicated. NTPs: ATP, CTP and GTP. (**D**) Occupancy of the A2c promoter by ternary complexes. Whole TP–DNA bearing ternary complexes formed in the presence of RNAP, ATP, CTP and GTP were cut with *Hin*dIII, as described in Materials and methods. The positions at which the DNA fragment containing the A2c promoter runs when free of ternary complexes and when occupied are indicated.

nascent RNA at this promoter (Elías-Arnanz and Salas, 1997). Due to lack of adequate restriction sites to obtain a single DNA fragment extending from the left TP end to the C1 promoter, experiments were performed using the whole ϕ29 genome (TP–DNA). The extent of C1 promoter occupancy in our reactions was estimated by gel retardation, as described in Materials and methods. Briefly, TP–DNA was first incubated with RNAP, ATP, CTP and GTP, then cleaved with *Eco*RI and *Ssp*I and finally subjected to agarose gel electrophoresis. About 42% of the TP–DNA molecules contained ternary complexes at C1, as estimated from the specific retardation of the 1260 bp band containing the C1 promoter in reactions containing RNAP and the three nucleotides (see Figure 3B). The C1 promoter lies 1674 bp from the right TP end and 17 611 bp from the left TP end. Using the complementary *Cla*I restriction fragment (*Cla*I A DNA), we reported previously that a ternary complex at C1 would block replication forks moving codirectionally, that is, from the right TP end (Elías-Arnanz and Salas, 1997). Here we show that the same ternary complex also blocks progression of replication forks moving head-on (Figure 3C). Thus, when TP–DNA bearing the ternary complex at C1 was replicated, stalling of the replication fork at a position that could only correspond to head-on collisions at C1 was observed (between the 19.2 kb full-length DNA and the 16.5 kb size marker). As expected, a band corresponding to replication fork stalling due to codirectional collisions with the ternary complex at C1 was also observed (between the 1.9 and 1.3 kb size markers). Although stable ternary complexes at other ϕ29 promoters were not expected to form at the concentration of RNAP and with the subset of nucleotides used, replication fork stalling at two additional positions was observed. The sizes of these two bands, ~14 and 5 kb long, could only correspond to codirectional and head-on collisions, respectively, with ternary complexes formed at A2c bearing a single nucleotide (note that expression of the A2b and A3 promoters is negligible under the conditions used). Although most ternary complexes with transcripts shorter than 10 nt are unstable (Carpousis and Gralla, 1985; Levin *et al*., 1987; Straney and Crothers, 1987; Krummel and Chamberlin, 1989), some reports have suggested that ternary complexes with very short transcripts can be stable (Schulz and Zillig, 1981). Gel retardation analysis showed that a stable ternary complex was indeed formed at A2c, since ~20% of the 759 bp band containing this promoter was shifted when RNAP, ATP, CTP and GTP were present in the reaction (Figure 3D). No shift was detected when RNAP alone was included in the reaction. As shown above for the *Cla*I B DNA fragment, the observed decrease in the yield of full-length DNA (74%) could be almost fully accounted for by the amount of stalled replication forks (68%). Thus, the simultaneous observation of replication fork arrest at two different promoters is due to incomplete occupancy of these promoters rather than to transient stalling of the replication fork. The differences in the amount of replication forks arrested due to head-on collisions and codirectional collisions at both promoters is most likely to stem from differences in the frequencies at which DNA replication is initiated at the two TP ends.

Arrested replication forks can be elongated when RNAP resumes transcription

We have shown previously that ϕ29 DNAP remains associated with the DNA template after encountering a codirectionally halted transcription ternary complex, and is able to continue DNA replication once the RNAP is allowed to move (Elías-Arnanz and Salas, 1997). To determine whether a similar situation applies for head-on collisions, we analysed the ability of the stalled replication products to be chased to full-length DNA after the RNAP resumes elongation.

We first performed the chase experiments using whole TP–DNA bearing transcription ternary complexes at C1 and A2c. This template DNA was replicated to generate stalled replication intermediates from head-on and codirectional collisions at both promoters. Then, the ribonucleotide (UTP) omitted to form the stable ternary complexes was added to allow translocation of RNAP. The transcription elongation step was performed in the presence of: (i) an excess of cold dATP (0.5 mM), to stop further incorporation of radioactive label into the replication

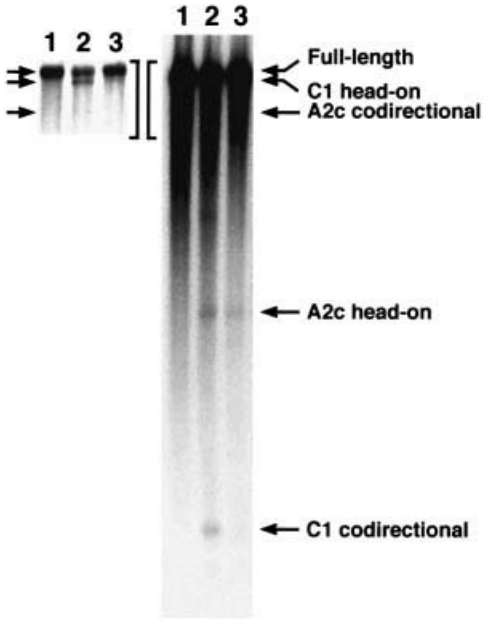

Fig. 4. Elongation of arrested replication forks upon resumption of RNAP movement when whole TP–DNA was used as template. Left panel: shorter exposure of the upper part of the gel shown in the right panel, so as to visualize elongation of stalled replication forks at C1 better. Lane 1 is a control reaction performed with naked whole TP–DNA. Lane 2 shows replication fork stalling in reactions with TP–DNA bearing ternary complexes at C1 and A2c. Lane 3, chase reaction of the arrested replication forks 10 min after RNAP was allowed to resume transcription by addition of UTP.

intermediates; (ii) rifampicin (50 μg/ml), to prevent formation of new ternary complexes; and (iii) excess M13 single-stranded DNA (ssDNA, 500 ng) or heparin (250 ng), which trap unbound DNAP molecules very efficiently. By blocking further incorporation of radioactive label into the DNA strands being synthesized, we can easily follow the fate of the stalled replication intermediates once transcription is allowed to resume. If the blockage on replication is relieved upon resumption of transcription, then the stalled bands will disappear, with a corresponding increase in the amount of full-length DNA. Neither of the trapping agents present in the amounts indicated destabilize replication forks or RNAP ternary complexes, but will sequester the DNAP and completely block replication when added to the reaction before the DNAP (data not shown). Thus, if the DNAP had dissociated from the template DNA as a result of the head-on collision, or were displaced from it after transcription resumes, the DNAP would be sequestered by the trapping molecules and the arrested replication forks would not be chased to longer products. As shown in Figure 4, ~90 and 50% of the replication forks stalled due to head-on collisions at C1 and A2c, respectively, disappeared when RNAP movement was permitted (compare lanes 2 and 3 for the specific bands). In agreement with our previous observations, elongation of the majority of the replication forks stalled due to codirectional collisions at C1 resumed when RNAP movement was allowed (Figure 4; Elías-Arnanz and Salas, 1997). Likewise, replication intermediates arising from codirectional collisions at A2c also disappeared after the chasing step. The decrease in the amount of stalled bands at the two promoters was paralleled by a

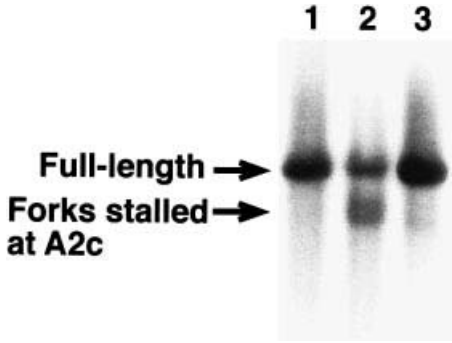

Fig. 5. Elongation of arrested replication forks upon resumption of RNAP movement when *Cla*I B DNA was used as template. Lane 1 is a control reaction performed with naked *Cla*I B DNA. Lane 2 shows replication fork stalling at A2c in reactions with *Cla*I B DNA bearing ternary complexes at this promoter. Lane 3, chase reaction of the arrested replication forks 1 min after RNAP was allowed to resume transcription by addition of CTP.

proportional increase in the amount of full-length DNA. This indicates that the DNAP elongates the stalled replication products by copying the same DNA strand it was using before transcription resumed. Our results argue against the DNAP switching template strands under the onslaught of the advancing RNAP to make fold-back DNA. If this were the case, the stalled replication intermediates should have been chased to products twice their original size: ~35 kb for head-on and 3 kb for codirectional collisions at C1, and ~10 kb for head-on and 28 kb for codirectional collisions at A2c. Bands corresponding to these sizes were never observed, even when the elongation step was performed for longer periods of time to ensure complete formation of the predicted larger products. We therefore conclude that the fate of the stalled replication bands after transcription resumes is their conversion into full-length DNA. Elongation of the nascent RNAs in the transcription ternary complexes was checked by primer extension (data not shown).

To rule out the possibility that resolution of head-on collisions requires two converging replication forks to collide with the same transcription ternary complex, a chase experiment like that described above was performed using the *Cla*I B DNA fragment (Figure 5). First, stable transcription ternary complexes were formed at the A2c promoter by omitting CTP. DNA bearing the halted transcription complexes was replicated for 5 min and, after taking a sample for monitoring replication stalling, CTP, cold dATP, rifampicin and a DNAP-trapping agent were added (see above). The reaction was allowed to proceed for 1 min with the additional components before a second sample was taken to follow the fate of the stalled band. After transcription elongation resumed, ~85% of the replication forks that had stalled at A2c disappeared, while the yield of full-length *Cla*I B DNA increased by the same amount (Figure 5, compare lanes 2 and 3). Since replication forks in the *Cla*I B DNA fragment can only move in one direction (from left to right), these results indicate that resolution of head-on collisions between DNAP and RNAP is intrinsic to the nature of the collision. The case of two oppositely oriented replication forks colliding with the same transcription ternary complex, a likely situation in replication of whole TP–DNA, might aid resolution of the collision but is not strictly required.

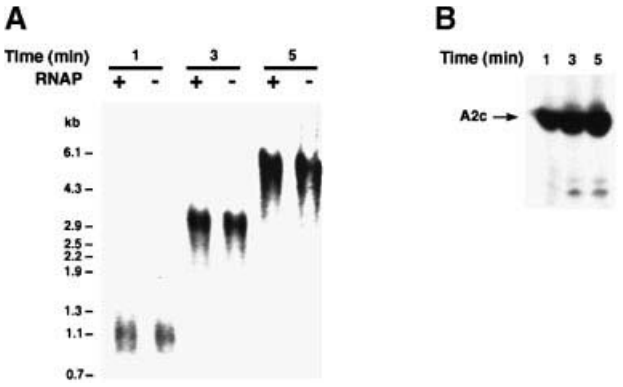

Fig. 6. Collisions between oppositely moving replication and transcription complexes. (**A**) Replication fork advance after 1, 3 and 5 min in reactions with or without actively transcribing complexes (± RNAP). On the left, the positions (kb) of several size markers are indicated. (**B**) Primer extension analysis showing expression of the A2c promoter in the *Cla*I B DNA fragment after 1, 3 and 5 min of concurrent transcription and replication.

In summary, the DNAP neither falls off the template upon encountering a halted transcription complex head-on, nor is it displaced when the RNAP is allowed to proceed with transcription. Once the RNAP initiates movement in the opposite direction, the DNAP resumes normal replication and continues to do so until it synthesizes full-length DNA.

Head-on collisions between concurrently moving DNA replication and transcription complexes

Our studies with halted transcription complexes revealed that ϕ29 DNAP moving head-on is unable to go past a static transcription ternary complex. However, head-on collisions were resolved when transcription elongation was allowed to resume. Encounters of the replication machinery with an already moving transcribing complex might then occur without negative consequences for either process. On the contrary, if collisions with actively transcribing RNAP molecules were not efficiently resolved, severe interference on replication would be expected to occur. To assess the consequences of such collisions, we followed replication of *Cla*I B DNA with and without concurrently moving transcription complexes. Replication initiation was synchronized by pre-incubating the template DNA and DNAP in reaction buffer lacking dCTP (see Materials and methods). With only dATP, dGTP and dTTP present, the DNAP stalls after incorporating the first eight nucleotides, thus having undergone the rate-limiting transition stage (Méndez *et al*., 1997). After the pre-incubation step, synchronous elongation of the 8 nt DNA primers was triggered by the addition of dCTP. At the same time, RNAP and poly(dI–dC) (as non-specific competitor DNA) were added to form actively transcribing complexes. The rate of replication fork advance was monitored by taking samples at different times (1, 3 and 5 min) during the reaction and running them next to size markers (Figure 6A). Transcripts produced at different times from the main promoter in the *Cla*I B DNA fragment, A2c, were analysed by primer extension, as described in Materials and methods (Figure 6B). Transcripts originating at the A2c promoter could interfere most with replication; this promoter is strong, and the transcripts generated from it can be as long as 5000 nt, although approximately half of the transcripts are shorter (~1100 nt) due to premature termination at the TA1 transcriptional terminator (this study; Barthelemy *et al*., 1987). The A2b promoter, located very close to the A2c promoter and that gives rise to transcripts of almost the same length, is at least 50 times weaker under our reaction conditions (not shown). The A1 promoter is a relatively strong promoter, but transcripts from this promoter are only 321 nt long (Barthelemy *et al*., 1987). Transcripts from the weak A1IV promoter are not detected under our reaction conditions (not shown). Thus, based on the above considerations, replication forks moving from the single TP end in the *Cla*I B DNA fragment would most often encounter RNAP molecules transcribing from the A2c promoter. Figure 6A shows that the replication forks advanced at the same rate (~20 nt/s) irrespective of whether the template DNA was being concurrently transcribed or not. From the results shown in Figure 6B, we estimated the percentage of DNA molecules that had initiated transcripts at the A2c promoter during the reaction: ~1 out of 10 molecules (10%) after 1 min, 1 out of 3.5 (30%) after 3 min and 1 out of 2 (50%) after 5 min. Of the 10% DNA molecules that initiate transcription at A2c each minute, half (5%) will bear transcripts that will stop at TA1, and the remaining half will bear transcripts that will continue to the end of the *Cla*I B DNA fragment. At a rate of advance of 20 nt/s, the RNAP will reach the TA1 terminator in ~1 min and the DNAP would take a little over 3 min to pass it (the TA1 terminator is located 3.8 kb away from the replication origin). Thus, of the percentage of DNA molecules that have initiated transcription at A2c after 3 min (30%), head-on collisions between DNAP and RNAP may occur in only about half of them (i.e. 15% for the half that will bear transcripts having gone past TA1). In contrast, head-on collisions are possible in all DNA molecules that initiate transcription at A2c during the last 2 min of reaction (20%). Overall, we estimate that head-on collisions will occur in ~35% of the replicated molecules, assuming random initiation of DNA replication (~15% in the DNA region before the TA1 terminator, plus 20% in the DNA region spanning from the transcription start site to the TA1 terminator). We reported previously that under conditions where neither replication nor transcription were synchronized, codirectional collisions between moving DNAP and RNAP will cause significant interference on replication fork advance (Elías-Arnanz and Salas, 1997). Under conditions of synchronous replication and, in agreement with our previous results, interference on replication fork advance was detected, even though only 10% of the DNA molecules being replicated were also being transcribed (data not shown). Thus, the percentage of DNA molecules that may be undergoing head-on collisions seems sufficiently high to have yielded detectable differences between reactions with and without concurrent transcription, if collisions were not readily resolved.

Discussion

DNA is the substrate for various enzymatic reactions. However, little is known of the rules that govern the trafficking of the protein complexes involved. The interaction between two fundamental processes for copying

information from DNA, like transcription and replication, could lead to one of several outcomes. A replication fork might be able to pass a transcribing RNAP, either leaving the ternary complex intact or knocking the RNAP off the template. Alternatively, the bulky RNAP might represent a 'road block' for an approaching replication fork. Upon codirectional collisions, the replication fork would be forced to move behind the RNAP, at the slower synthesizing rate of RNAP; in contrast, head-on collisions could lead to a stalemate, blocking the further movement of both protein complexes.

In this paper, we present evidence that halted transcription complexes of *B.subtilis* σ^A RNAP completely block progression of ϕ29 DNAP moving head-on. Replication fork arrest appears to be caused by direct physical collision with the stalled ternary complexes, as sharp bands mapping very close to the location of the ternary complexes were observed. Upon collision, the DNAP remained bound to the DNA template, being unable to move unless the transcription complex was allowed to translocate. In contrast to a ternary complex, non-transcribing RNAP molecules did not interfere with replication fork progression. The difference probably lies in the weaker interactions that characterize promoter binding by RNAP, as compared with a stable ternary complex. Our results with halted transcription complexes are very similar to those that we observed in the case of codirectional collisions (Elías-Arnanz and Salas, 1997) and differ from those previously reported using the T4 *in vitro* replication system. In this latter system, a mechanism exists that permits the replication apparatus to bypass stalled *E.coli* RNAP transcription complexes after codirectional or head-on collisions (Liu *et al*., 1993, 1994; Liu and Alberts, 1995). Although head-on collisions with a ternary transcription complex caused replication fork arrest in ϕ29, the stalled replication products could be extended to full-length DNA once the RNAP resumed elongation. This observation implies that at least one of the two machineries involved must have the capacity to pass over the other, so that polymerization can be resumed in two opposite directions. Since the main difference between the two situations is the state of the transcription complex, either halted or moving, it is likely that the RNAP plays a major role in the resolution of head-on collisions with the ϕ29 replication machinery. The ability of elongating RNAP to undergo significant conformational changes is likely to be a determining factor (Roe *et al*., 1985; Krummel and Chamberlin, 1992a,b; Das, 1993; Chamberlin, 1994; Wang *et al*., 1995). The fact that transcription need not be resumed for resolving head-on collisions with the T4 replication system points to the replication machinery having a more active role in the resolution mechanism, in contrast to the situation in ϕ29. In this regard, the strand-displacement capacity of the complete T4 replication apparatus could play an important role in its ability to pass a transcription complex efficiently. Thus, the replication fork will stall for minutes after its encounter with an oppositely oriented ternary complex when the T4 DNA helicase is omitted (Liu and Alberts, 1995). A lower global strand-displacement capacity of ϕ29 DNAP, which works as a monomeric enzyme, compared with the complete T4 replication apparatus containing a helicase, may explain its inability to bypass a stalled transcription complex. DNA duplex opening by the movement of actively transcribing RNAP could conceivably favour resolution. As discussed below, head-on collisions between a moving replication fork and an active transcription complex would very much resemble a head-on collision between two replication forks, a situation that ϕ29 DNAP is successful at resolving.

Our studies of codirectional collisions between replication and transcription using the *Cla*I A DNA fragment led us to envisage the following situation. Upon encountering a moving RNAP, the DNAP would move behind the RNAP at the lower speed that characterizes transcription until the RNAP leaves the template (Elías-Arnanz and Salas, 1997). This model was proposed based on our results with stalled ternary complexes and on the observation of a 50% reduction in the replication rate of actively transcribed DNA. This reduction was considered to be particularly significant, given that collisions with actively transcribing complexes could only occur in ~14% of the length of the *Cla*I A DNA molecule, mainly with transcripts from the C2 promoter (which span only 1.8 kb in a DNA fragment whose total size is 13 kb). Thus, the existence of a mechanism to resolve codirectional collisions seemed unlikely. In our analysis of head-on collisions between concurrent replication and transcription, we find that the replication rate of *Cla*I B DNA is not affected by the presence of actively transcribing complexes. Considering that the estimated percentage of DNA molecules undergoing head-on collisions is ~35%, the observed lack of interference in replication fork advance argues for the existence of a resolution mechanism. These results are in good agreement with our observation that replication forks stalled from colliding head-on with a static transcription ternary complex will engage in normal elongation once the RNAP is allowed to move.

A unique feature of the ϕ29 system is its symmetrical mode of replication without lagging strand synthesis: DNA replication proceeds continuously from both ends by strand displacement, generating two types of replicative intermediates (see Figure 7A). Transition from type I to type II replicative intermediates involves a head-on collision between the two replication forks, which ϕ29 DNAP is able to resolve successfully. The strong affinity of ϕ29 DNAP for ssDNA (Blanco *et al*., 1989) may help undergo this transition, inasmuch as the same kind of interactions with DNA could be maintained in both replicative types. The only difference would be that in type I intermediates its ssDNA-binding domain would contact the strand that the DNAP is displacing, whereas in type II intermediates it would contact the template strand. In this respect, the situation at the leading edge of RNAP in head-on collisions with the replication fork would resemble a transition from type I to type II replicative intermediates. This similarity may contribute favourably to resolving head-on collisions with transcription complexes. The existence of a mechanism to resolve collisions between a replication fork and a transcription complex moving head-on could have additional advantages in the achievement of efficient replication of ϕ29 DNA *in vivo*. A collision between three complexes like the one depicted in Figure 7B is a likely situation during the life cycle of bacteriophage ϕ29: (i) a replication fork that initiated, for instance, at the right replication origin, is moving at a lower speed due to collision with RNAP transcribing codirectionally; (ii) before RNAP terminates transcription, it encounters a replication fork head-on that initiated at the opposite replication origin; (iii)

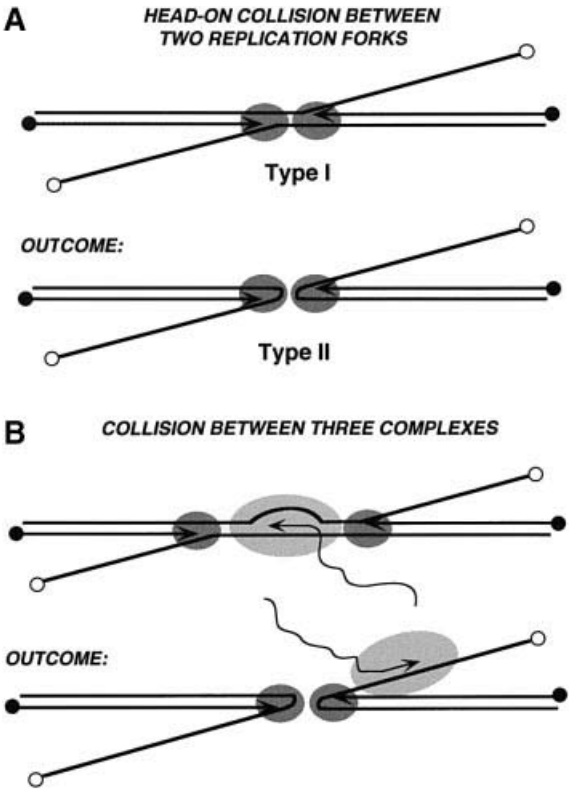

Fig. 7. (**A**) A type I replicative intermediate with the two replication forks coming close to each other is illustrated. ϕ29 DNAP is able to resolve this collision generating type II replicative intermediates. The DNAP molecule is shown maintaining similar interactions with the DNA in both replicative types. (**B**) A replication fork that initiated at the right replication origin is shown moving behind a transcription complex with whom it collided codirectionally. Before the RNAP leaves the template, a second collision occurs with a replication fork moving from the opposite genomic end. Resolution of the head-on collision between DNAP and RNAP indirectly leads to resolution of the codirectional collision between DNAP and RNAP. DNAP is represented in dark grey, RNAP in light grey and TP as full or empty circles.

indirectly, resolution of the head-on collision with RNAP also helps resolve the codirectional collision, when transition from type I to type II replication intermediates occurs. The RNAP may remain attached to its original template strand upon resolution, as depicted in Figure 7B. Alternatively, the transcription ternary complex may switch templates without loss of the transcript, an ability that has been reported for the *E.coli* RNAP (Liu and Alberts, 1995; Nudler *et al*., 1996). The occurrence of the contemplated resolution events would be of particular significance in the case of collisions with transcription complexes originated at the A2c promoter and the A3 promoter, whose transcripts can be as long as 5000 and 12 000 nt, respectively. In addition to the above considerations, spatial and/or temporal separation of transcription and replication may contribute to perform these two fundamental processes efficiently in ϕ29-infected cells (see Elías-Arnanz and Salas, 1997).

Materials and methods

Reagents

Bacillus subtilis σ^A RNAP was purified by the method of Sogo *et al*. (1979). ϕ29 DNAP and TP were obtained from overproducing *E.coli* strains (Zaballos *et al*., 1989; Lázaro *et al*., 1995). Unlabelled nucleoside triphosphates (ultrapure) and deoxynucleoside triphosphates were from Pharmacia Biotech. [α-^{32}P]dATP (3000 Ci/mmol) and [α-^{32}P]ATP (3000 Ci/mmol) were from Amersham International. Replication-competent TP–*Cla*I B DNA was obtained as described (Elías-Arnanz and Salas, 1997). Briefly, ϕ29 TP–DNA (isolated as described in Peñalva and Salas, 1982) was digested with the restriction enzyme *Cla*I, loaded onto a sucrose gradient (10–40% w/v), and centrifuged at 30 000 r.p.m. for 24 h at 4°C. After centrifugation, fractions containing the TP–*Cla*I B DNA fragment were pooled, washed extensively with 10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0 at 4°C and concentrated to ~100 ng/μl using a Microcon microconcentrator.

Replication of DNA bearing transcription ternary complexes

To obtain templates for DNA replication bearing halted transcription ternary complexes, 1 nM of either TP–*Cla*I B or whole TP–DNA was incubated for 5–10 min at 37°C in 25 μl of reaction buffer [50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 30 mM ammonium sulfate, 1 mM dithiothreitol (DTT), 4% glycerol, 0.1 mg/ml bovine serum albumin (BSA)] with 3.5 nM *B.subtilis* σ^A RNAP and a limited subset of NTPs (200 μM each); 20 μM each dNTP and 0.2 μM [α-^{32}P]dATP (2 μCi) were also included for the subsequent replication reaction. Before replication was initiated by the addition of 20 nM preformed TP–DNAP complex (Blanco *et al*., 1992), ternary complex formation was stopped with rifampicin (50 μg/ml). Replication reactions were performed at 30°C for the times indicated, after which they were stopped by the addition of EDTA (10 mM) and SDS (0.1%). Unincorporated nucleotides were removed by spinning in Sephadex G-50 columns containing 0.1% SDS. The products were analysed by alkaline agarose gel electrophoresis and autoradiography. The percentage of full-length DNA and stalled replication forks was estimated by densitometric scanning of the radiolabelled bands. Values obtained for replication of naked DNA were arbitrarily assigned a value of 100%. The percentage of replication forks stalled at a given position was calculated as follows: using the values obtained by densitometric scanning, we estimated the amount of full-length DNA that would have been produced if replication had not arrested at that position (correcting for the size of the band relative to the size of full-length DNA). This value (expressed as a percentage relative to the replication of naked DNA) is used as a measurement of the amount of stalled replication forks. Ternary complex formation and stability was followed as described (Elías-Arnanz and Salas, 1997).

Replication of DNA bearing actively transcribing complexes

Reactions were done in reaction buffer (50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 30 mM ammonium sulfate, 1 mM DTT, 4% glycerol, 0.1 mg/ml BSA). Replication initiation was synchronized by incubating for 30 min at 30°C the following components (in 25 μl reaction buffer): 1 nM TP–*Cla*I B DNA, 10 μM dATP, 10 μM dGTP, 10 μM dTTP, 0.2 μM [α-^{32}P]dATP (2 μCi), 6 nM DNAP, 20 nM TP, 200 μM each of all four NTPs, RNasin ribonuclease inhibitor (7.5 U). The last two components are only relevant during the next step of the reaction, after RNAP is added. The transition from protein-primed initiation into DNA-primed elongation during ϕ29 DNA replication is the rate-limiting step of the reaction and has been shown to occur between nucleotides 6 and 9 (Méndez *et al*., 1997). In the presence of only dATP, dGTP and dTTP, the DNAP will synthesize an 8-nt DNA primer (5′-AAAGTAAG-3′). A 30 min incubation period under the stated conditions will allow a sufficient number of DNAP molecules to undergo the transition stage so that elongation proceeds synchronously once the missing deoxynucleotide (dCTP) is added. After this step, dCTP (10 μM), 40 nM σ^A-RNAP and 2 μg of the non-specific competitor DNA poly(dI–dC) were added to allow replication elongation and the synthesis of transcripts. For the concentration of nucleotides supplied in the reaction, both DNAP and RNAP advance at approximately the same rate (~20 nt/s). Samples were taken at 1, 3 and 5 min after the addition of the components indicated to monitor the rate of replication and to estimate the yield of transcripts. Replication was followed by alkaline agarose gel electrophoresis. The production of transcripts was estimated by primer extension analysis as described in Monsalve *et al*. (1995). The oligonucleotides (20 pmol) used to hybridize specifically with the transcripts were labelled at their 5′ end with [γ-^{32}P]ATP and polynucleotide kinase so that only one labelling molecule per transcript was introduced during the primer extension reaction. The products were analysed by 8 M urea–6% polyacrylamide gel electrophoresis and autoradiography. The values obtained by densitometric scan of the signals corresponding to specific transcripts were compared with those obtained for spots where known numbers of [γ-^{32}P]ATP molecules had been deposited. These values were used to estimate the number of transcript molecules produced.

Then, the number of DNA molecules present in the reaction was divided by the number of transcript molecules produced to estimate the percentage of DNA molecules bearing a transcript.

Promoter occupancy

Whole TP–DNA or TP–*Cla*I B DNA, treated as described above to form ternary complexes, and control reactions lacking RNAP or NTPs were performed in parallel. To determine occupancy of the A2c promoter by ternary complexes, the DNA was digested with 10 U of *Hin*dIII for 10 min at 37°C and the resulting fragments were separated by gel electrophoresis. Occupancy of A2c was followed by retardation of the 759 bp restriction fragment containing this promoter (as well as the A2b and A3 promoters). Digestion with *Hin*dIII was not suitable to follow occupancy of the C1 promoter (when whole TP–DNA was used) due to the larger size of the DNA fragment containing this promoter (2498 bp) and the proximity of other restriction fragments in the gel. Thus, to determine occupancy of the C1 promoter by ternary complexes, whole TP–DNA was digested with 10 U each of *Eco*RI and *Ssp*I for 10 min at 37°C. The C1 promoter falls in a 1260 bp restriction fragment whose retardation due to promoter occupancy can now be followed. In this case, the A2c promoter lies in a restriction fragment that is too large (9860 bp) to detect its occupancy by gel shift. After digestion, glycerol (3% final concentration) was added to each sample. Gel electrophoresis was performed in 1% NuSieve agarose gels in 1× TBE buffer (89 mM Tris–borate, 89 mM boric acid, 2.5 mM EDTA) for 4–6 h at 6 V/cm and room temperature. The bands were visualized by ethidium bromide staining.

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