Bacteriophage ϕ 29 DNA replication arrest caused by codirectional collisions with the transcription machinery

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The consequences on replication of collisions between ϕ 29 DNA polymerase, a monomeric replicase endowed with strand displacement capacity, and the transcription machinery have been studied *in vitro*. Codirectional collisions with stalled transcription ternary complexes at four different promoters in the ϕ 29 genome were found to block replication fork progression. Upon collision, the DNA polymerase remained on the template and was able to resume elongation once the RNA polymerase was allowed to move. Collisions with RNA polymerase molecules moving in the same direction also interfered with replication, causing a decrease in the replication rate. These results lead to the proposal that in bacteriophage ϕ 29 a transcription complex physically blocks the progression of a replication fork. We suggest that temporal regulation of transcription and the low probability that the replication and transcription processes colocalize *in vivo* contribute to achieving minimal interference between the two events.
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Introduction

DNA replication and transcription are two fundamental processes that can occur simultaneously on the same DNA molecule, leading to eventual collision between the two machineries. Evaluating the impact of such events on the processivity of each polymerization reaction is of great interest. The outcome of the encounter may initially depend on the orientation of the transcription unit with respect to the replication fork. In general, the available information from *in vivo* and *in vitro* studies suggests that head-on collisions are more disadvantageous for the cell than codirectional collisions. In *Escherichia coli*, the orientation of the transcription units in the chromosome seems to have evolved to avoid head-on collisions between RNA polymerase (RNAP) and replication complexes; thus, most of the highly active genes are replicated and transcribed in the same direction (reviewed in Brewer, 1988). In agreement with this notion, electron microscopy analysis in *E. coli* revealed that, when replication and transcription do occur in opposite directions, replication fork progression is reduced (French, 1992). *In vivo* studies with several eukaryotic organisms demonstrated the presence of polar replication fork barriers downstream of 35S

rRNA genes; as a result, most ribosomal DNA is replicated unidirectionally by forks moving in the direction of transcription, and head-on collisions between the replication and transcription complexes are prevented (Brewer and Fangman, 1988; Linskens and Huberman, 1988; Little *et al.*, 1993; Wiesendanger *et al.*, 1994). In addition, *in vivo* transcription-dependent stalling of replication forks when they oppose the direction of transcription, but not when they are codirectional, have been mapped to tRNA genes of *Saccharomyces cerevisiae* (Deshpande and Newlon, 1996). The only available results from *in vitro* studies have been obtained using the highly purified T4 replication system. Surprisingly, a replication fork was found to pass through a codirectionally located transcription complex without displacing it, leaving intact its ability to resume RNA chain elongation (Liu *et al.*, 1993). Similar results have been obtained using moving RNAP molecules (Liu *et al.*, 1994). In comparable studies involving head-on collisions, the replication fork was able to pass a transcription complex poised to move in the opposite orientation after a pause of a few seconds. Consistent with head-on collisions being more disadvantageous, this pause was longer than that observed for codirectional collisions (Liu and Alberts, 1995). Despite this brief pause, the T4 replication apparatus was able to go past a transcription complex. Such a resolving mechanism requires the involved machineries to have certain capabilities that may not apply in other systems. Another system suitable for assessing *in vitro* the outcome of such collision events is that of bacteriophage ϕ 29 of *Bacillus subtilis*, which differs in several respects from the T4 system. A special characteristic of the linear and double-stranded (19 285 bp) ϕ 29 genome is the presence of a terminal protein (TP) covalently linked to each 5' end which is required for replication. Unit length ϕ 29 DNA can be synthesized efficiently *in vitro* using a minimal system consisting of highly purified TP, DNA polymerase and TP-containing ϕ 29 DNA (TP-DNA) as template (Blanco and Salas, 1985; Blanco *et al.*, 1989). A unique feature of ϕ 29 DNA replication that distinguishes it from other replication systems, including that of T4 bacteriophage, is its symmetrical mode of replication. The existence of two replication origins located at the ends of the genome and the high processivity and strand-displacement abilities of the monomeric ϕ 29 DNA polymerase allow both strands to be replicated continuously (reviewed in Blanco and Salas, 1996).

The arrangement of genes in the ϕ 29 genome reflects their temporal regulation: all late genes are clustered in the central part of the genome and are transcribed from the A3 promoter, whereas early genes are located at both ends of the genome and are transcribed from several promoters on the complementary strand (reviewed in Salas and Rojo, 1993; see Figure 1A). Due to this arrangement

Fig. 1. Transcriptional units in phage $\phi 29$. (A) Arrangement of transcription units in the $\phi 29$ genome. The terminal protein (TP) is shown covalently linked to each 5' end. The direction of transcription from each promoter is indicated by arrowheads. Filled arrowheads indicate transcription terminators (TA1, TB1, TB2 and TD1). (B) The two fragments generated by cleavage with *ClaI*, fragment A (13 140 bp) and fragment B (6145 bp), are shown. The transcription initiation sites of the different promoters relative to the TP-DNA end in each *ClaI* fragment are also given. (C) The longest RNA sequences that can be obtained from each promoter when transcription is initiated with a subset of only three NTPs. When the underlined nucleotides are omitted, the RNAP halts at the previous position.

of the transcription units and the fact that replication starts from both DNA ends, any replication fork will probably encounter transcription complexes moving along the template in the same or in the opposite orientation. For instance, a replication fork moving from the left end of the genome may undergo head-on collisions with RNA polymerase molecules transcribing early genes, whereas if starting from the right end it will encounter early transcription complexes codirectionally. Then, the arrangement of genes in $\phi 29$ does not clearly reflect a situation that evolved to avoid one kind of collision rather than the other, as has been proposed for other systems. In this study, we examine the effect on $\phi 29$ DNA replication of codirectional collisions between the replication and transcription machineries. Detailed studies on the effects of head-on collisions on $\phi 29$ DNA replication will be reported elsewhere (M.Eliás-Arnanz and M.Salas, in preparation). In contrast to the results obtained using the T4 *in vitro* system, our analysis in $\phi 29$ shows that both stalled and moving codirectional transcription complexes interfere with progression of the replication fork.

Results

Advance of the replication fork is blocked upon collision with a codirectionally-stalled transcription ternary complex

To study the consequences of codirectional collisions independently of head-on collisions, we used $\phi 29$ TP-DNA treated with the restriction enzyme *ClaI* as DNA template. Cleavage with *ClaI* is convenient because the two fragments generated (A and B) contain promoters which are oriented in opposite directions (see Figure 1A and B); also, since replication of $\phi 29$ DNA starts only at

TP-linked $\phi 29$ DNA ends (replication origins), by cutting the DNA we make sure that the replication fork moves in only one direction along the template DNA. To obtain separated *ClaI* fragments which retain an optimal ability to replicate, the parental TP molecule in the DNA fragment must remain intact; this requirement imposes certain restrictions on the conditions to which the DNA can be exposed, such that standard techniques for gel isolation of DNA are not applicable. Sucrose density gradient sedimentation proved a suitable method for purifying replication-competent *ClaI* fragments (see Materials and methods). The direction of transcription from the promoters in the *ClaI* fragment A (C2, C1, B2 and B1) is the same as the direction of replication of this DNA fragment (Figure 1A and B). Thus, the consequences of codirectional collisions between the two polymerases were studied using this fragment as template DNA. Our first approach was to analyze the outcome of collisions between a replication fork and a stalled transcription complex. RNAP ternary complexes halted at a particular point in transcription can be obtained by providing the RNAP with a limited subset of NTPs. As shown in Figure 2A, in the presence of only ATP, UTP and GTP, transcription complexes initiated at the C2 promoter bearing the expected 17 nt RNA (see Figure 1C) were formed. The observed ternary complex was a stable transcription complex, as judged by its ability to be elongated when the missing NTP was provided (Figure 2A). With the subset of NTPs provided, no stable ternary complexes were formed at the C1, B2 and B1 promoters with nascent transcripts of only 3, 1 and 5 nt, respectively. Replication was performed on DNA templates containing either the halted 17 nt ternary complex or mock-treated DNA, and the products were analyzed by polyacrylamide gel electrophoresis. Transcription from the C2 promoter starts 160 bp from the TP-DNA end (replication origin) in the *ClaI* fragment A; if the DNA polymerase were unable to pass the ternary complex, we would expect the replication fork to stop at a position close to the promoter. As shown in Figure 2B, stalling of about 81% of replication intermediates 158 nt from the end was observed only with the template DNA bearing the transcriptional ternary complex (% of stalling was calculated as described in Figure 2 legend). As a consequence, the yield of full-length DNA was reduced to 13%. These results suggest that the replication fork cannot pass a codirectionally stalled RNAP.

To generalize our findings, we also analyzed the outcome of codirectional collisions with ternary complexes at three additional promoters: the C1, B2 and B1 promoters in the *ClaI* fragment A. To form stable ternary complexes at any of these three promoters, a different subset of NTPs was used. By withholding UTP, instead of CTP, ternary complexes with longer transcripts at the C1 (14 nt), B2 (29 nt) and B1 (8 nt) promoters can be formed; with this subset of nucleotides, no stable ternary complex would form at the C2 promoter, with a nascent RNA only 4 nt long (Figure 1C; see also Krummel and Chamberlin, 1989). Figure 3A shows the products from replication reactions with DNA bearing ternary complexes formed at different RNAP concentrations in the presence of ATP, CTP and GTP. As control, replication reactions with DNA bearing no ternary complexes were performed (that is,

Fig. 2. Codirectional collisions with a ternary complex halted at the C2 promoter. (A) Lane 1 indicates the position of the 17 nt labeled nascent RNA produced when transcription initiated at the C2 promoter halts because of omission of CTP. Lane 2, 90 s chase (after addition of CTP, at 30°C) of the 17 nt ternary complex, formed in the presence of 2 nM RNAP. A transcript smaller than the nascent 17-mer appearing during the chase may be a cleavage product of the 17 nt RNA, as has been observed in other studies (Liu and Alberts, 1995). The samples were analyzed by 8 M urea–20% polyacrylamide gel electrophoresis. (B) Replication of free TP-DNA *Cla*I fragment A (lane 1) or TP-DNA *Cla*I fragment A bearing the 17 nt ternary complex at C2 (formed in the presence of 2 nM RNAP) (lane 2). The positions corresponding to full-length replicated DNA and to stalling of the replication fork at C2 (158 nt from the TP-DNA end) are indicated. The samples were run in an 8 M urea–6% polyacrylamide gel. In this gel, full-length *Cla*I fragment A (13 140 nt) is not well resolved from long replication intermediates which have not yet reached full-length. This is particularly obvious in lane 1, where the yield of full-length and long replication intermediates is significantly higher than in lane 2, thus appearing as a broad band. The same replication reaction run in an alkaline agarose gel shows a major band corresponding to full-length DNA and a smear corresponding to incomplete replicated molecules. The percentage of full-length DNA and stalled replication forks was estimated by densitometric scanning of the radiolabelled bands. Numbers given are relative to the values obtained for replication of free DNA, which is arbitrarily assigned a value of 100%. The percentage of stalled forks 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 the indicated position (correcting for the size of the band relative to the size of full-length DNA). This value (expressed as percentage relative to replication of free DNA) is an estimation of the percentage of stalled replication forks.

DNA preincubated with the different RNAP concentrations in the absence of NTPs). Stalling of replication at a position that mapped to the C1 promoter was observed at all RNAP concentrations used. Stalling of replication at positions mapping to the weaker promoters B2 and B1 was detected only at the highest RNAP concentrations used (mostly at 7 nM and 14 nM, about a 4-fold and an 8-fold excess relative to promoters, respectively). The lack of a band corresponding to stalling of replication at C2 (data not shown) was as expected, since under these conditions no stable ternary complexes form at the C2 promoter. Two additional pieces of evidence support the suggestion that the observed bands represent collisions with the stalled ternary complexes. First, the bands were observed only when both RNA polymerase and the three ribonucleotides were present in the reaction; in the absence of ribonucleotides, the bands were not seen (Figure 3A).

Fig. 3. Analysis of codirectional collisions with ternary complexes at the C1, B2 and B1 promoters. (A) Replication reactions performed with *Cla*I fragment A preincubated with different concentrations of RNAP (0–14 nM) with or without NTPs (ATP, CTP and GTP). The replication products were analyzed by alkaline agarose gel electrophoresis. The positions of full-length DNA and of replication forks stalled at each promoter are indicated. (B) Occupancy of the different promoters determined in parallel by gel shift assays (after 4 h electrophoresis at room temperature in a 1.5% NuSieve agarose gel), as described in Materials and methods. The same conditions (RNAP concentration and presence/absence of NTPs) hold for each lane in panel A and the lane directly below in panel B. The positions of the DNA fragments containing each promoter are indicated.

Second, if rifampicin (an inhibitor of transcription initiation) was added to the reactions prior to formation of the ternary complexes, rather than after the transcription complexes had already formed, replication proceeded without any stops (not shown). Promoter occupancy at the different RNAP concentrations was determined in parallel, as described in Materials and methods. As shown in Figure 3B, no specific shift was observed in reactions lacking NTPs, in which no ternary complexes would form. At the highest concentrations used (7 and 14 nM), all the bands were slightly shifted (even bands that did not contain any promoter) due to the nonspecific binding of RNAP. At these RNAP concentrations, a decrease in the yield of full-length DNA was observed; it is likely that the interference with replication is due to nonspecific binding of RNAP to the TP-DNA ends, thus competing for the binding of the TP-DNA polymerase complex (see Figure 3A and Table I). This interference was efficiently overcome by the addition of 20-fold excess of the non-specific competitor DNA, poly(dI–dC) (data not shown). However, in the reactions shown in Figure 3, poly(dI–dC) was not included because it competes for binding of RNAP to the weak promoters B1 and B2, making detection of stalling at these two promoters difficult. Furthermore, the smear caused by ethidium bromide staining in the presence of such an excess of poly(dI–dC) also hampered a reliable determination of promoter occupancy. Nevertheless, in reactions containing NTPs (ATP, CTP and GTP), a specific shift of the DNA fragment which contained the C1 promoter was observed at all the RNAP concentrations

Table I. Distribution of stalled replication forks at increasing concentrations of RNA polymerase and correlation of replication stalling with promoter occupancy

RNAP (nM)	DNA without ternary complexes ^a	DNA bearing ternary complexes ^b				
	Full-length	Full-length	Stalling at C1	Stalling at B2	Stalling at B1	C1 occupancy
0.0	100	100	0	0	0	0
0.8	100	55	43	0	0	40
1.7	100	36	60	0	0	55
3.5	81	20	60	0	4	60
7.0	60	8	57	4	6	60
14.0	38	3	59	9	6	60

^aNumbers represent the percentage of full-length *Clal* fragment A obtained in replication reactions performed in the absence of NTPs and with increasing amounts of RNA polymerase, relative to replication reactions without RNA polymerase. Numbers were estimated by densitometric scanning of the data in Figure 3A.

^bNumbers in the first four columns represent the percentage of full-length *Clal* fragment A (relative to the percentage in the absence of RNAP) and replication forks stalled due to collisions with ternary complexes at the C1, B2 and B1 promoters, estimated by densitometric scanning of the data in Figure 3A. The percentage of stalling at each promoter was calculated as described in the legend to Figure 2. The last column shows the percentage of *Clal* fragment A molecules with ternary complexes at the C1 promoter, determined by gel retardation assays in agarose gels as described in Materials and methods. Numbers were obtained by densitometric scanning of the unshifted and shifted bands in Figure 3B. No occupancy data for the B1 and B2 promoters are given, since occupancy of the B1 and B2 promoters was below the detection limits, as discussed in the text.

used. The percentage of replication forks stalled at this promoter correlated with its occupancy in all cases (see Figure 3 and Table I). Moreover, at the lowest RNAP concentrations (0.8 and 1.7 nM), at which stalling was detected only at C1 and no interference due to nonspecific binding of RNAP occurs, the decrease in the yield of full-length DNA could be fully accounted for by the amount of replication forks stalled at C1 (i.e. the proportion of full-length DNA and of replication forks stalled at C1 totalled about 100%). At these RNAP concentrations, no shift of the fragments containing the weaker B1 and B2 promoters was observed, as expected. Even at the highest RNAP concentrations used, when stalling at the three promoters was detected, promoter occupancy at C1 was no greater than 60%. At this RNAP concentration (14 nM), no specific shifts for the fragments containing the B1 and B2 promoters were observed; a specific shift for the longer fragment containing the B1 promoter was also not detected at lower agarose concentrations and longer running times than those specified in Figure 3B (not shown). Rather, the same general shift seen in reactions performed in the absence of NTPs appeared (Figure 3B), indicating that these promoters are not occupied in the majority of the DNA molecules. It should be noted that our detection limit for occupancy of the C1 promoter is about 10% and, from the replication data, occupancy of the B1 and B2 promoters would be expected to fall below or close to this detection limit. In agreement with the lack of stable ternary complexes at the C2 promoter under these conditions, no specific shift of the DNA fragment containing this promoter was observed at any of the RNAP concentrations used. Thus, the fact that we are able to detect stalling at the C1, B2 and B1 promoters in the same reaction (at 14 nM RNAP) is due to incomplete occupancy of all the promoters in the DNA template molecules, rather than to complete occupancy and transient stalling of the replication fork at each ternary complex encountered. Our studies of four different ϕ 29 promoters therefore indicate that a stalled transcription ternary complex physically blocks the progress of a replication fork moving in the same direction.

The DNA polymerase is stalled behind the RNA polymerase in codirectional collisions

The above results show that incomplete replication products are synthesized when the replication and transcription machineries collide. As a consequence of such collisions, the DNA polymerase may remain associated with the template or it may fall off the template. To distinguish between these two possibilities, we determined whether the incomplete replication products could be chased to full-length DNA once the RNAP was allowed to move by adding the omitted ribonucleotide. Replication reactions were performed using as template the *Clal* fragment A containing stalled ternary complexes at the C1, B2 and B1 promoters; then, the transcription complexes were challenged to move by adding UTP. At the same time, we added an excess of nonradioactive dATP (0.5 mM) to chase labelled replication intermediates, M13 single-stranded DNA (500 ng) to trap any DNA polymerase molecule that was not loaded on the template, and rifampicin (50 μ g/ml) to ensure that no new ternary complexes were formed. The fate of the replication intermediates was analyzed by alkaline agarose gel electrophoresis, and production of the corresponding transcripts was followed by primer extension analysis. After transcription was allowed to resume, we were able to detect transcripts from the ternary complexes, showing that they are not dead-end complexes in which elongation is blocked, but true transcription intermediates (data not shown). As shown in Figure 4, samples of the replication assays taken before the fourth NTP was added showed the expected pausing at the C1, B2 and B1 promoters (time 0). Two minutes after transcription was allowed to resume, the majority of the stalled replication intermediates had extended to longer products, and after 4 min, most of them had been converted to full-length DNA. Although a small fraction of the replication forks stalled at the B1 promoter remained (probably representative of a fraction of inactive ternary complexes), the complete disappearance of those stalled at C1 and B2 strongly supports the conclusion that when codirectional collisions occur the

Fig. 4. Chase of stalled replication products obtained due to codirectional collisions. In the replication reaction using TP-DNA *ClaI* fragment A shown in this figure, the ternary complexes were not preformed by incubation at 37°C for 10 min; instead, the transcription reaction to form ternary complexes and the replication reaction were allowed to proceed simultaneously for 12 min at 30°C. Under these conditions, the yield of stalled products is slightly lower (and, consequently, the yield of full-length DNA is slightly higher) than in reactions using TP-DNA *ClaI* fragment A with preformed ternary complexes. Samples were taken before (time 0) and 1–4 min after the RNAP was allowed to resume transcription by addition of UTP. The products were analyzed by alkaline agarose gel electrophoresis.

DNA polymerase remains stalled behind the RNA polymerase and can resume replication once the RNAP starts moving.

Codirectional concurrent replication and transcription

Since a halted ternary complex may not be functionally equivalent to one that is actively transcribing (Surratt *et al.*, 1991; Krummel and Chamberlin, 1992a,b), we have also investigated the effect of codirectionally moving RNAP molecules on replication. We have estimated that in our *in vitro* system the RNAP moves along the template four times more slowly than the DNA polymerase (10 nt/s for RNAP compared with 40 nt/s for DNA polymerase; see Materials and methods). This implies that a DNA polymerase is likely to overtake a more slowly moving RNAP. If such encounters are also disadvantageous for the DNA polymerase, as shown above for collisions with stalled ternary complexes, a decrease in the rate of replication would be expected to occur. The rate of replication was therefore determined in the presence and absence of concurrently moving transcription complexes using the *ClaI* fragment A. Reaction mixtures were prepared as described in Materials and methods. Samples were taken at different times during the reaction and run in alkaline agarose gels next to size markers to follow replication and estimate the rate of polymerization (Figure 5A). All reactions were performed in the presence of excess nonspecific competitor DNA to eliminate possible interference with replication by nonspecific binding of RNAP. Nevertheless, as an additional control, we compared replication reactions performed in parallel in the absence of NTPs and with or without RNAP. Transcription from the promoters contained in each fragment was determined by primer extension analysis. As shown in Figure 5B, of the four promoters located on the *ClaI*

Fig. 5. Replication and transcription complexes moving concurrently and codirectionally. (A) Analysis of products obtained at different times during replication of DNA molecules undergoing concurrent transcription (+RNAP, +NTPs) or not (–RNAP, +NTPs). As additional controls, samples were taken after 8 min replication performed in the absence of NTPs and in the presence or absence of RNAP (last two lanes). The positions of size markers run in parallel are indicated. Samples were run in alkaline agarose gels. (B) Primer extension analysis showing production of transcripts from the four promoters in the *ClaI* fragment A in the presence of concurrent replication.

fragment A, the C2 promoter is the strongest one. This promoter is located 160 bp from the right end of the φ29 DNA and is the first promoter that the DNA polymerase would encounter when replicating this DNA fragment. Transcripts initiated at the C2 promoter proceed through the C1 promoter and terminate at the transcriptional terminator TD1, reaching a size of ~1850 nt (Barthelemy *et al.*, 1987) (Figure 1A and B). The C1 promoter is not as strong as the C2 promoter (Figure 5B), is located 1674 bp from the end and gives rise to a transcript of ~340 nt that terminates also at TD1. The B2 promoter (3979 bp from the right end) and the B1 promoter (at position 7825) are very weak (Figure 5B) and seem to give rise to short transcripts (Barthelemy *et al.*, 1987). Given the relative strength of the promoters and the length of the transcripts, transcription from the C2 and C1 promoters would be expected to interfere most with replication. As shown in Figure 5A, when RNA polymerase molecules were moving in the same direction along the DNA, the rate of replication slowed down from about 40 nt/s to 20 nt/s. In agreement with this, if the replication intermediates obtained at any particular time were chased to full-length DNA (after adding excess unlabeled dATP, M13 single-stranded DNA and rifampicin), the yield of *ClaI* fragment A in the presence of concurrent transcription was half the yield obtained in its absence (not shown). No interference with replication was observed when RNAP but no NTPs were included in the reaction (compare samples taken after 8 min in reactions performed with or without RNAP in the absence of NTPs). This indicates that, in reactions including both RNAP and NTPs, collisions with actively transcribing RNAP molecules are the cause of the observed interference. A considerable proportion of the replication intermediates in reactions with concurrent transcription were smaller than

1800 nt. As mentioned above, transcription from the strong C2 promoter starts 160 bp from the end and proceeds up to the TD1 terminator at positions 2016–2022. Therefore, we believe that the accumulation of replication intermediates in this region is due to collisions with RNAP molecules mostly transcribing from the C2 promoter. It is also likely that interference caused by collisions with this specific transcription complex (giving rise to the longest transcript and initiated at the strongest promoter in *Clal* fragment A) contributes greatly to the final outcome.

Discussion

It is highly probable that the replication and transcription machineries sometimes encounter each other. Since these collisions may have negative consequences on either process, the cell has probably developed strategies to deal with such events. A biologically viable solution could be that the passage of the replication fork simply knocks aside the RNAP from the template. However, results from *in vivo* experiments suggest that the replication complex cannot do this (see Brewer, 1988). A different strategy might be avoidance of collisions by separating the two events spatially, temporally or both. Thus, the spatial separation of adenovirus replication and transcription sites (Pombo *et al.*, 1994) may have evolved partly to prevent the two processes interfering with one another. Similarly, studies with human bladder carcinoma cells indicate that transcription domains and replication domains do not overlap (Wansink *et al.*, 1994). A precise arrangement of the directions of transcription and replication would help to prevent disadvantageous collisions. A nonrandom gene organization of the transcription units has been observed in *E.coli*, several *E.coli* phages [e.g. T7, ϕ X174, λ and plasmid ColE1 (reviewed in Brewer, 1988)], as well as in the *B.subtilis* chromosome (Zeigler and Dean, 1990): most of the heavily transcribed genes are found to be oriented in the same direction as the replication fork, suggesting that head-on collisions are avoided. In addition, mechanisms may have developed to resolve collisions without any negative consequence for either process. The only direct evidence for such a mechanism comes from recent experiments using *in vitro* T4 replication: the replication fork was able to pass either a codirectional or a head-on transcription complex, allowing both replication and transcription to be completed after the collision. Selection for the appropriate orientation of genes with respect to replication is difficult in T4 due to the use of multiple modes to initiate replication throughout the infection cycle (reviewed in Mosig and Colowick, 1995). In this context, the development of a resolving mechanism represents an obvious advantage. The genome organization of bacteriophage ϕ 29 has also not evolved to avoid collisions of either kind (see below). However, in contrast to the T4 system, we find that codirectional collisions between the ϕ 29 DNA polymerase and the transcription machinery block replication fork progression. In addition, experiments in progress indicate that a transcription complex also interferes with the advance of the replication complex when the two complexes collide head-on (M.Eliás-Arnanz and M.Salas, in preparation). The difference in behaviour of ϕ 29 and T4 is probably a consequence of differences

between the two replication systems. In T4, the high processivity required for DNA replication is achieved by the association of the catalytic subunit with accessory proteins; in addition, several other proteins are required for efficient leading and lagging strand synthesis (see Kornberg and Baker, 1992; Nossal *et al.*, 1995). In this regard, the highly processive T4 DNA helicase (the gene 41 protein) is necessary for the ability of the T4 replication machinery to bypass a transcription complex (Liu and Alberts, 1995). A special feature of ϕ 29 DNA polymerase is its intrinsically high processivity and the ability to carry out DNA polymerization coupled to strand-displacement in the absence of any accessory proteins and DNA helicases; although these two properties are well designed to support the characteristic symmetrical mode of replication of ϕ 29 DNA (reviewed in Blanco and Salas, 1996), they are clearly insufficient to confer the DNA polymerase the ability to pass a transcription complex. Since ϕ 29 DNA polymerase works as a monomeric enzyme it is unlikely that the presence *in vivo* of other proteins not included in our assay could alter the conclusions of our *in vitro* experiments. Nevertheless, we cannot entirely rule out this possibility. Has ϕ 29 developed a strategy for performing these two processes with minimal interference *in vivo*? As mentioned above, the transcription units in ϕ 29 DNA do not appear to be arranged so as to avoid collisions of either kind (codirectional or head-on). All late genes are transcribed from the same DNA strand and the early genes (located at the two ends of the genome) are transcribed from the complementary strand (see Figure 1; reviewed in Salas and Rojo, 1993). Since the two ϕ 29 DNA ends are active replication origins and replication proceeds continuously from one end to the other, both codirectional and head-on collisions are possible for a given transcription complex depending on the end from which the replication fork proceeds. Nevertheless, the temporal regulation of transcription in ϕ 29, which leads to a temporal regulation of replication, may contribute to decreasing the likelihood of collisions between the replication and transcription machineries. Early transcription drives expression of genes essential for DNA replication; thus DNA replication begins only after transcription from the early A2b, A2c and C2 promoters occurs. In addition, as a result of early transcription, the regulatory protein p4 and high amounts of the early protein p6 are produced. Both proteins may help prevent collisions with early transcription complexes. On the one hand, in the presence of p6 the strong C2 promoter and the weak C1, B2 and B1 promoters are rapidly turned off (Whiteley *et al.*, 1986; Barthelemy *et al.*, 1989; our unpublished results). Moreover, based on the estimated intracellular concentration of p6 and its binding cooperativity to DNA, it is conceivable that *in vivo* a fraction of the TP-DNA molecules form a compact nucleoprotein complex with p6 (Gutiérrez *et al.*, 1994). Such p6-covered TP-DNA molecules, although they could be replicated, would be difficult to transcribe (our unpublished results), thereby diminishing the probability that the two processes colocalize. The regulatory protein p4, on the other hand, would help decrease the likelihood of collisions by repressing the two main early promoters A2b and A2c (Barthelemy and Salas, 1989; Rojo and Salas, 1991; Monsalve *et al.*, 1995, 1996). Protein p4 also activates transcription from

CODIRECTIONAL COLLISION BETWEEN DNA POL AND RNAP COMPLEXES

DNA pol slows down

Fig. 6. A replication fork is shown approaching a transcription complex moving in the same direction but more slowly. After the encounter, the DNA polymerase (dark grey) adjusts its polymerization rate, moving behind the RNAP complex (light grey). The arrows above the replication and transcription complexes indicate the direction of their movement; differences in arrow length indicate different polymerization rates.

the A3 promoter (Sogo *et al.*, 1979), which gives rise to the structural components of the phage particle and proteins involved in morphogenesis and cell lysis. Late transcription might not be readily compatible with concurrent replication, inasmuch as transcription from the A3 promoter extends over 12 kb, giving a wide margin for interference. However, there is evidence from studies *in vivo* (Monsalve *et al.*, 1995) suggesting that p4 activates transcription from the A3 promoter in only a fraction of the TP-DNA molecules present at the middle and late times of infection. The existence of these two TP-DNA populations again reduces the chances of collisions with transcription complexes.

Our analysis of codirectional collisions with stalled ternary complexes reveals replication blocks which map fairly close to the location of the ternary complexes. In our most accurate analysis using the ternary complex at C2, we observe a 158 nt band for collisions with a transcription complex initiated at position 160 with a 17 nt nascent RNA (Figure 2). These results are consistent with the arrest of the replication fork being caused by direct physical collisions between the two complexes. Chase experiments of stalled replication forks suggest that in codirectional collisions the DNA polymerase remains on the DNA behind the transcription complex, and is unable to move unless the transcription complex also moves. In addition, we have shown that when both replication and transcription machineries move simultaneously on DNA, the rate of replication slows down. This observation can be interpreted by considering: (i) our results with stalled ternary complexes, which indicate that the DNA polymerase is unable to go past a stalled transcription complex; (ii) the difference in the polymerization rates between the two enzymes. Our view on how codirectional collisions interfere is thus as follows (see Figure 6): following a collision, the replication complex would slow down, synthesizing at the slower rate characteristic of the RNAP until the RNAP left the template and allowed the replication fork to proceed at its normal rate.

In summary, our results provide *in vitro* evidence that in bacteriophage φ29, where genome organization has not evolved to prevent a specific type of collision, codirectional collisions between the replicating and transcribing machineries interfere with replication fork progression. We suggest that temporal regulation of transcription and the low probability of replication and transcription colocalizing *in vivo* help minimize interference between the two events.

Materials and methods

Nucleotides and proteins

Unlabelled nucleoside triphosphates (ultrapure) and deoxynucleoside triphosphates were purchased from Pharmacia Biotech. [α - 32 P]dATP (3000 Ci/mmol) and [α - 32 P]ATP (3000 Ci/mmol) were obtained from Amersham International. φ29 DNA polymerase and TP were overproduced in *E. coli* cells and purified as previously described (Zaballos *et al.*, 1989; Lázaro *et al.*, 1995). *B. subtilis* σA RNAP was obtained as described by Sogo *et al.* (1979); in the preparations used in this study, about 66% of the enzyme contained the σA subunit. RNAP concentrations are given considering only σA-containing RNAP.

DNA templates

φ29 TP-DNA was isolated as described previously (Peñalva and Salas, 1982). Purified TP-*Cla*I DNA fragments functionally active in replication were obtained as follows. φ29 TP-DNA (30 μg) was digested with the restriction enzyme *Cla*I, which generates two fragments: B (6145 bp) and A (13 140 bp). The fragments were separated by sucrose density gradient centrifugation. The digested φ29 TP-DNA (30 μg) was loaded on to an 11 ml sucrose gradient (10–40% w/v in 1 M NaCl, 0.1 M Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0) prepared in a Beckman polyallomer centrifuge tube (14×95 mm). Centrifugation was performed at 30 000 r.p.m. for 24 h at 4°C in a Beckman TST41.14 swinging bucket rotor. After centrifugation, 0.5 ml fractions were collected by puncturing the bottom of the tubes. Aliquots from each fraction were run in an agarose gel (after treatment with proteinase K to remove the TP) to determine which fractions contained the *Cla*I fragment A. Fractions containing this DNA fragment were pooled, washed extensively (with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0 at 4°C) to eliminate the sucrose and salt, and concentrated using a Microcon microconcentrator 30 (Amicon). The DNA in the concentrated samples was quantified by ethidium bromide fluorescence.

DNA replication assays

The buffer used for the replication and transcription reactions (also referred to as reaction buffer) was as follows: 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 30 mM ammonium sulfate, 1 mM dithiothreitol, 4% glycerol, 0.1 mg/ml bovine serum albumin. In this buffer, both the replication and transcription reactions are efficient and can be performed sequentially (to study replication of DNA bearing ternary complexes) or simultaneously (to study concurrent replication and transcription). The ionic strength used was defined by the requirements of the replication reaction. Although the transcription reaction works well at higher ammonium sulfate concentrations, the replication reaction becomes rapidly inhibited. φ29 TP-DNA fragments bearing ternary complexes were obtained after incubation for 10 min at 37°C in 25 μl of reaction buffer containing 1 nM of TP- DNA *Cla*I fragment A, 0.8–14 nM *B. subtilis* σA-RNAP, a limited subset of NTPs (at a concentration of 200 μM each) as well as 20 μM of each dNTP and 0.2 μM [α - 32 P]dATP (2 μCi) for the subsequent replication reaction. To prevent formation of any new halted ternary complexes, after the incubation period at 37°C, rifampicin was added to a final concentration of 50 μg/ml. Replication was then triggered by adding 20 nM of preformed TP-DNA polymerase complex prepared as previously described (Blanco *et al.*, 1992) and incubating at 30°C (the replication reaction is more efficient at 30°C than at 37°C). The reactions were stopped after 12 min by adding EDTA (10 mM final concentration) and SDS (0.1% final concentration). Replication of DNA bearing ternary complexes was compared with replication of mock-treated DNA which had also been kept at 37°C for 10 min in reaction buffer (containing a limited subset of NTPs as well as each dNTP and [α - 32 P]dATP) prior to replication, but in the absence of RNAP. After the replication reaction, the samples were filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS to remove

unincorporated nucleotides and the Cerenkov radiation of the excluded fraction was then determined. To check that the right ternary complexes were being formed in the above reactions, [α - 32 P]ATP was also included to obtain labelled nascent RNAs during the 10 min incubation period at 37°C. The subset of NTPs used was either: (i) GTP (200 μ M), UTP (200 μ M) and [α - 32 P]ATP (0.2 μ M); or (ii) GTP (200 μ M), CTP (200 μ M) and [α - 32 P]ATP (0.2 μ M). After incubation at 37°C, reactions were stopped by adding formamide loading buffer and labelled transcripts were analyzed by 8 M urea–20% polyacrylamide gel electrophoresis. The stability of the ternary complexes was tested by following the ability to survive gel filtration in Sephadex G-50 spin columns equilibrated in reaction buffer (50 mM Tris–HCl, pH 7.5, 10 mM MgCl₂, 30 mM ammonium sulfate, 1 mM dithiothreitol, 4% glycerol). In addition, the ability of the ternary complex to elongate was tested, as follows: after formation of the ternary complex, rifampicin (50 μ g/ml) and an excess (1 mM) of unlabeled ATP were added. An aliquot from the reaction was then chased with 200 μ M of the missing NTP and incubation at 30°C; the ability of the nascent RNA to be elongated was checked by 8 M urea–20% polyacrylamide gel electrophoresis.

Analysis of promoter occupancy by gel retardation

To determine whether the amount of replication forks stalled after collision with a particular ternary complex correlates with RNAP occupancy of that promoter, we performed gel retardation assays of ϕ 29 TP-DNA fragments bearing ternary complexes obtained as described above (after 10 min incubation at 37°C in 25 μ l reaction buffer containing 1 nM of TP-DNA *Cla*I fragment A, 0.8–14 nM σ A-RNAP, a limited subset of NTPs as well as each dNTP). As controls, similar reactions but without RNAP or NTPs were performed in parallel. After the incubation period at 37°C, rifampicin was added to a final concentration of 50 μ g/ml and the samples were incubated at 30°C for 12 min, to mimic the treatment of samples undergoing replication. Then, to analyze promoter occupancy of the various promoters, the DNA was simultaneously digested with 10 U each of *Eco*RI, *Ssp*I and *Nco*I for 10 min at 37°C (these three enzymes work efficiently in the replication–transcription reaction buffer). After digestion with these three restriction enzymes, the C2 promoter is in a 653 bp fragment, the C1 promoter in a 1260 bp fragment, the B2 promoter in a 779 bp fragment and the B1 promoter in a 2074 bp fragment. After digestion, glycerol was added to each sample (3% final concentration) and the samples were loaded in 1–2% NuSieve agarose gels. The gels were run in 1 $\times$ TBE (89 mM Tris base, 89 mM boric acid, 2.5 mM EDTA) at 6 V/cm for 4–6 h at room temperature and the bands were visualized by staining with ethidium bromide.

Concurrent replication and transcription assays

Concurrent replication and transcription reactions were performed in 25 μ l of reaction buffer (50 mM Tris–HCl, pH 7.5, 10 mM MgCl₂, 30 mM ammonium sulfate, 1 mM dithiothreitol, 4% glycerol, 0.1 mg/ml bovine serum albumin) containing 1 nM TP-DNA *Cla*I fragment A, 2 μ g nonspecific competitor poly(dI–dC), 14 nM σ A-RNAP, 200 μ M each NTP, 20 μ M each dNTP, 0.2 μ M [α - 32 P]dATP (2 μ Ci) and 20 nM preformed TP-DNA polymerase complex. Transcription and replication were simultaneously initiated by adding RNAP and the preformed TP-DNA polymerase complex and then incubating at 30°C. To determine the DNA elongation rates in these experiments, samples were taken at different times and the DNA replication products were analyzed by alkaline agarose gel electrophoresis in the presence of size markers. The average size of the newly synthesized DNA was estimated after densitometric scanning of the autoradiograms. Likewise, the rate of RNA synthesis under these conditions was determined in parallel reactions including one radiolabeled NTP. Production of transcripts was also determined by primer extension analysis, as described previously (Monsalve *et al.*, 1995).

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