

Phage P4 DNA replication *in vitro*

Ramón Díaz Orejas¹, Günter Ziegelin, Rudi Lurz and Erich Lanka*

Max-Planck-Institut für Molekulare Genetik, Abteilung Schuster, Ihnestr. 73, Dahlem, D-14195 Berlin, Germany and ¹Centro de Investigaciones Biológicas (CSIC), C/ Velázquez 144, E-28006 Madrid, Spain

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ABSTRACT

Phage P4 DNA is replicated in cell-free extracts of *Escherichia coli* in the presence of partially purified P4 α protein [Krevolin and Calendar (1985), *J. Mol. Biol.* 182, 507–517]. Using a modified *in vitro* replication assay, we have further characterized this process. Analysis by agarose gel electrophoresis and autoradiography of *in vitro* replicated molecules demonstrates that the system yields supercoiled monomeric DNA as the main product. Electron microscopic analysis of *in vitro* generated intermediates indicates that DNA synthesis initiates *in vitro* mainly at *ori*, the origin of replication used *in vivo*. Replication proceeds from this origin bidirectionally, resulting in θ -type molecules. In contrast to the *in vivo* situation, no extensive single-stranded regions were found in these intermediates. The initiation proteins of the host, DnaB and DnaG, and the chaperones DnaJ and DnaK are not required for P4 replication, because polyclonal antibodies against those polypeptides do not inhibit the process. The reaction is inhibited by antibodies against the SSB protein, and by ara-CTP, a specific inhibitor of DNA polymerase III holoenzyme. Consistent with previous reports, P4 *in vitro* replication is independent of transcription by host RNA polymerase. Novobiocin, a DNA gyrase inhibitor, strongly inhibits P4 DNA synthesis, indicating that form I DNA is the required substrate.

INTRODUCTION

P4 is a temperate satellite bacteriophage of *E. coli* which relies on functions of bacteriophage P2, that delivers the capsid and tail components and the proteins required for the cell lysis. P4 itself does not encode information for such functions. P4 DNA (11,624 bp) is stored in the phage heads as a double-stranded linear molecule containing 19-base cohesive ends. Following P4 phage infection, P4 DNA circularizes. The prophage state is either maintained by integration into the host chromosome, or by autonomous replication of the multicopy plasmid (1, 2). Initiation of P4 plasmid replication requires the product of the P4 α gene ($gp\alpha$), which is multifunctional with specific DNA binding, primase and 3'→5' helicase activities (3). In addition,

two *cis* acting elements on P4 DNA are essential: the origin of replication (*ori*) and the *cis* replication region (*crr*), both containing type I repeats (4). $gp\alpha$ binds specifically to those iterons (3). *crr* cannot function as an origin, but is required for initiation from *ori* (4). Rounds of DNA synthesis initiated at *ori* proceed to the terminus either bidirectionally or alternatively unidirectional. *In vivo* replicative intermediates originated at *ori* correspond to the θ -type mode of replication and contain frequently long single-stranded regions at the growing fork, that are deployed in a *trans* configuration (5). *In vivo* analysis indicated that P4 replication is independent of DnaA, DnaB, DnaC, and DnaG, but requires DNA polymerase III holoenzyme (6).

Insight into the process of replication, the roles of the $gp\alpha$ and the interactions between this protein and the host replication proteins can be gained by *in vitro* analysis. Such a system has been developed, based on extracts of P4 infected cells and partially purified $gp\alpha$ (5, 7). In this system, P4 replication depended strictly on $gp\alpha$, SSB, and DNA polymerase III holoenzyme. The reaction was resistant to rifampicin as well as to nalidixic acid, an inhibitor of DNA gyrase. Using partially purified $gp\alpha$ and DNA polymerase III holoenzyme, and purified SSB, the main product was relaxed monomeric DNA. *In vitro* replication was initiated probably at *ori*, because in the presence of chain terminators, synthesized DNA was labeled preferentially in the *ori* region.

In order to further characterize the P4 replication process *in vitro*, we have improved the P4 replication assay previously described (7). Here we present *in vitro* data on the origin and direction of replication, the products, and the requirement for several host proteins. This system will be useful for the biochemical characterization of the initiation/elongation and termination stages of P4 DNA replication, and also to explore functional relations between $gp\alpha$ and components of the host replication machinery.

MATERIAL AND METHODS

Bacterial strains and plasmid

E. coli K-12 strain C600 (8) was used to prepare plasmid-free extracts. SCS1(pMS4 Δ 1) overproducing $gp\alpha$ (9), was used to prepare extracts enriched in $gp\alpha$. WM1288 (10) carrying the

* To whom correspondence should be addressed

dnaK756 mutation, was the source for DnaK deficient extracts. P4 plasmid DNA used in the *in vitro* replication assays was obtained as previously described (9) from P4vir1, an immunity insensitive P4 phage (11).

Preparation of cell-free extracts

0–40% ammonium sulfate fractions were obtained from cell-free extracts prepared essentially as described (12). The concentration of gp α in the extracts was evaluated as follows: After electrophoresis of samples containing extracts of the gp α overproducer or defined amounts of purified gp α on a denaturing polyacrylamide gel (0.1% SDS), the proteins were transferred electrophoretically to a nitrocellulose membrane and reacted for 2 h at 25°C with an appropriate dilution of a gp α -specific antiserum raised in a rabbit. A subsequent incubation for 45 min in a dilution (1:50) of dichlorotriazinyl amino fluorescein-conjugated goat anti-rabbit IgGs (Dianova) served to visualize the primary reaction (13). The fluorescence was measured using a Fluorimager 575 (Molecular Dynamics). The signals of the samples containing known amounts of purified gp α were used to determine the gp α content of the extract.

Assay of DNA replication

Standard reaction mixtures (25 μ l) contained the following components: HEPES-KOH (pH 8.0), 25 mM; KCl, 100 mM; EDTA, 0.25 mM; Mg(CH₃COO)₂, 20 mM; DTT, 1 mM; ATP, 4 mM; CTP, GTP and UTP, each 0.1 mM; dATP, dCTP and dGTP, each 40 μ M; [³H]dTTP, 10 μ M at a specific activity of 500 cpm/pmol; NAD, 25 μ M; cAMP, 25 μ M; creatine phosphate, 3 mM; creatine kinase, 0.1 mg/ml; polyethylene glycol 6.000, 2.5%; P4 DNA, 50 μ g/ml; gp α , 8 ng/ml. *E. coli* proteins of a 0–40% ammonium sulphate fraction were used (7). This extract contained endogenous NTPs and dNTPs in concentrations rate limiting for replication. The ATP regenerating system was absolutely required. The mixtures were incubated for 50 minutes at 30°C unless otherwise indicated. *In vitro* replication of mini R1 (pKN177, ref. 14) and of ColE1 was carried out using 70% ammonium sulphate fractions as described (15). DNA synthesis was measured by determining incorporation of labeled deoxynucleotide into acid-insoluble material (16). Polyclonal antisera against gp α , DnaB, DnaG, DnaJ and SSB were obtained from rabbits immunized with preparations of the purified proteins. When antisera were included in the assays, the antigen-antibody reaction was allowed to occur for at least 20 min at 4°C in the absence of DNA. To prevent unspecific inhibition, a maximum of 3 μ l serum was added to the standard mixture.

RESULTS

P4 DNA replication *in vitro* is gp α dependent

A 0–40% ammonium sulfate fraction prepared from the gp α overproducing strain SCS1 (pMS4 Δ 1) did not support P4 DNA replication *in vitro*, probably due to high amounts of gp α present in the extract. Therefore, we used aliquots of this extract to complement a 0–40% ammonium sulfate fraction of the plasmid-free strain C600 resulting in efficient P4 replication. The yield of product was optimal at 8–10 ng gp α /ml (Fig. 1A). Higher concentrations were strongly inhibitory. The reaction required Mg²⁺ ions and was optimal at approximately 20 mM Mg²⁺ and at 80–100 mM K⁺. Replication was dependent on P4 DNA; the optimal temperature was close to 35°C (data not shown). At

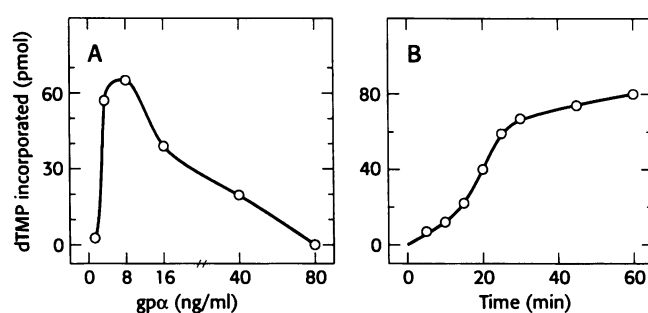


Figure 1. (A) gp α dependence of P4 DNA replication. The total incorporation of dTMP into DNA is plotted as a function of increasing gp α concentrations. (B) Kinetics of P4 DNA synthesis at 35°C. Independent assays were stopped at different times by the addition of EDTA to 50 mM. Samples were maintained at 4°C until the last one was taken.

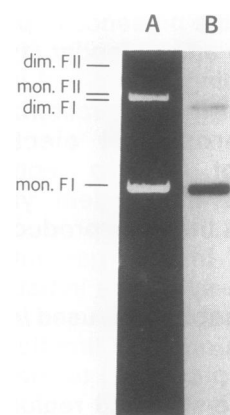


Figure 2. Analysis of P4 DNA replication products by gel electrophoresis. The replication assay was performed in the presence of [α -³²P]dATP. DNA was electrophoresed on a 0.7% agarose gel. Lane A: ethidium-bromide stained substrate DNA; lane B: autoradiograph of the replication products labeled with ³²P-dTTP. The monomeric and dimeric species of supercoiled covalently closed (form I) DNA and the open circular (form II) DNA are marked.

this temperature, replication was twice as efficient than at 30°C. To measure the rate of the reaction, samples were taken at several time points. The course of synthesis was sigmoidal. Within the first 10 min, the amount of product increased slowly. A strong increase was observed during the time period of incubation between 10 and 30 min. After 50–60 min, the synthesis reached saturation (Fig. 1B).

P4 DNA synthesized *in vitro* is supercoiled monomeric

To analyze the products of P4 *in vitro* replication, the standard reaction was performed in the presence of [α -³²P]dATP. The DNA synthesized was electrophoresed on an agarose gel and visualized by autoradiography (Fig. 2). The main product is monomeric form I DNA, accompanied by a minor fraction of monomeric form II DNA. Also a small amount is dimeric supercoiled, probably due to substrate DNA dimers. This result demonstrates, that *in vitro* one complete round of replication takes place, including the resolution of the daughter molecules and transformation into form I DNA.

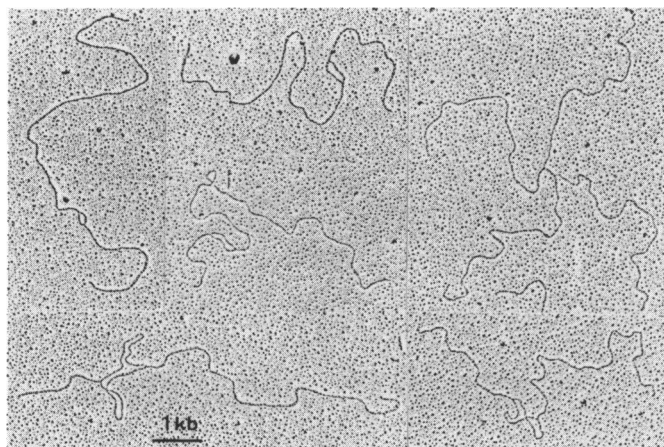


Figure 3. Electron microscopy of P4 *in vitro* replicative intermediates. Replicative intermediates were obtained by limited *in vitro* DNA synthesis in the presence of 10 μ M ddTTP. The reactions were run for 50 min at 30°C and terminated by the addition of EDTA to 50 mM. To prevent branch migration, the DNA was cross-linked with psoralen (17). To improve the efficiency of this process, the salt concentration was reduced to 20 mM by a 4-fold dilution in water. For cross-linking, 200 μ l of the sample were incubated three times with 10 μ l 2,5,9-trimethylpsoralen (1 mg/ml in DMSO) on ice for 5 min, each followed by 15 min irradiation with 366 nm UV-light. Subsequently, the intermediates were purified essentially as described (18) with slight modifications (19). The molecules were linearized with *Sma*I or *Pvu*II and then prepared for electron microscopy as described (20). Representative types of replicating molecules linearized with *Sma*I are shown.

To shift the equilibrium between form I and form II substrate DNA to form II, prior to the addition of gp α the reaction mixture was incubated with the DNA gyrase-specific inhibitor novobiocin. The resulting relaxed P4 DNA was not replicated demonstrating that supercoiled DNA is the required substrate as previously shown (7).

P4 DNA is replicated *in vitro* by the θ -type mode starting at *ori*

Replicative intermediates were generated *in vitro* by limited reaction in the presence of the chain terminator ddTTP (i) to ascertain that P4 *in vitro* replication was initiated at *ori*, (ii) to analyze the structure of replicating molecules, and (iii) to obtain information on the directionality of the process. The structure of the products was frozen by crosslinking both strands of double-stranded DNA with psoralen. Following linearization with a suitable restriction endonuclease, the intermediates were analyzed after visualization in the electron microscope (Fig. 3). Most of them contained a bubble of double-stranded replicated DNA flanked by the unreplicated parental strands. Another type was double-Y-shaped, with both branches of replicated DNA in the double-stranded form. These molecules originate from intermediates, in which the replication forks passed the recognition site of the restriction enzyme used for linearization. No intermediates with extensive single-stranded regions were found. However, a few molecules with short single-stranded segments at the replication forks were present in the preparation. These single-stranded regions probably represent the length of Okazaki-fragments generated by discontinuous DNA synthesis. This results demonstrate, that *in vitro* P4 DNA is replicated by the θ -type mode and that lagging strand synthesis is initiated frequently. Replicating molecules obtained in the presence or

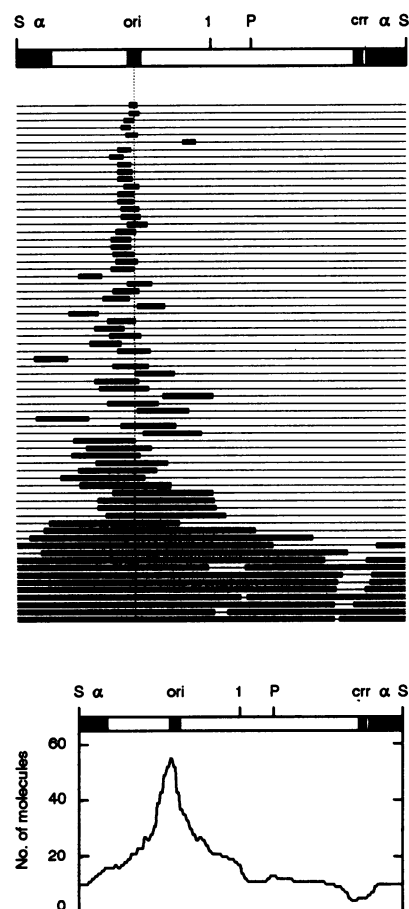


Figure 4. Upper panel: Line diagrams of partially replicated P4 DNA. The length of the replicated and unreplicated regions of 72 *Sma*I linearized DNA molecules similar to those shown in Fig. 3 were measured. The molecules are aligned in order of increasing extent of replication with the short unreplicated region to the left using a self-written program. The boxes represent the replication bubble. The corresponding linear map of the P4 genome (11,624 bp) is shown at the top of the figure. The black boxes represent *ori* and *crr*, the hatched regions mark the α gene. Restriction sites are; S, *Sma*I; P, *Pvu*II. Lower panel: Origin and direction of P4 DNA *in vitro* replication. The figure represents the set of data used in the upper panel. The most frequent position of small replication bubbles defines the origin of P4 replication. The corresponding linear map of the P4 genome is concordant to that of the upper panel.

absence of a DnaG-specific antiserum were indistinguishable and occurred with similar frequency (data not shown). Therefore, DnaG does not play a significant role in initiation or in elongation.

To determine the directionality of the process and the region, from which synthesis starts, intermediates linearized with *Sma*I or *Pvu*II were aligned with the bubble or the more extended branch of the double-Y-structure oriented to the left (Fig. 4). The analysis showed that replication predominantly was initiated at *ori*. Some molecules were found with a bubble located between *ori* and *crr* and some others with one corresponding to the 5' region of the α gene or to *crr*. Replication initiated from *ori* was probably bidirectional, because both forks of the bubble proceeded along the DNA. In most of the extensively replicated molecules, the remaining unreplicated segment is located at or around *crr* (Fig. 4). This indicates, that the reaction might terminate at a position (*ter*) expected for symmetrical bidirectional

Table 1. Protein requirements for P4, R1 and ColE1 DNA synthesis *in vitro*

Addition/Omission		Yield (%)		
(+)	(-)	P4	R1	ColE1
Complete		100	100	100
+ anti-DnaB serum	(0.2 μ l)	92	<5	6
	(1.0 μ l)	70	<5	5
+ anti-DnaG serum	(2.0 μ l)	43	<5	35
	(3.0 μ l)	31	<5	35
+ anti-SSB serum	(2.0 μ l)	<5	<5	30
	(3.0 μ l)	<5	<5	30
+ anti-gp(serum	(1.0 μ l)	27	nt	nt
	(2.0 μ l)	9	nt	nt
-DnaKa ^a		100	5	100

^a - DnaK indicates the use of a DnaK deficient extract prepared from WM1288. nt: not tested

replication. However, some intermediates were also present, in which termination occurred at other positions indicating an asymmetric bidirectional movement of the forks.

Host replication initiation proteins are not required *in vitro* for P4 replication

To identify some of the host proteins participating in the *in vitro* P4 replication, we investigated the effects of specific inhibitors of replication initiation proteins of the host (antibiotics or polyclonal antisera) on P4 DNA synthesis, and the ability of an extract deficient in DnaK to replicate P4 (Table 1). The reaction was sensitive to novobiocin at 35°C and at concentrations known to inhibit DNA gyrase, demonstrating that this topoisomerase is involved. However at 30°C, a residual activity of approximately 20% was detected (data not shown). This synthesis was probably due to a small fraction of supercoiled DNA still present in the assay, when initiation occurred. To confirm this possibility, P4 form I DNA was incubated with the standard replication mixture containing novobiocin without gp α at 30°C and 35°C, and then subjected to gel electrophoresis. During incubation at 35°C, all supercoiled DNA was converted into form II, which could not serve as a substrate, whereas at 30°C remaining form I DNA was detected. ara-CTP, an inhibitor of the α -subunit of DNA polymerase III, reduced the yield of product to 30% (data not shown). This result confirms the expectation that DNA polymerase III holoenzyme elongates the nascent chains. Rifampicin, inhibiting RNA polymerase, did not influence the reaction.

To evaluate the antisera experiments, replication of plasmids R1 and ColE1 served to verify the specificity of the sera (Table 1). Antibodies prepared against the primosomal DnaB protein had no effect on P4 DNA synthesis in our *in vitro* system at a concentration sufficient to block R1 and ColE1 replication. Antibodies against the host primase DnaG reduced the yield of product to approximately 40%, while R1 synthesis, which requires DnaG, was prevented. Incubation of the extract with a SSB specific antiserum abolished the P4 replication. DnaJ, a chaperone required to generate the P1 initiation complex (21), was dispensable, because a DnaJ specific antiserum did not influence P4 DNA synthesis (data not shown). An extract of WM1288 defective in the chaperone DnaK, which is essential to replicate R1 and P1 (22, 21), supported the P4 specific reaction.

These data indicate that SSB and DNA gyrase are essential in addition to polymerase III holoenzyme to bring about

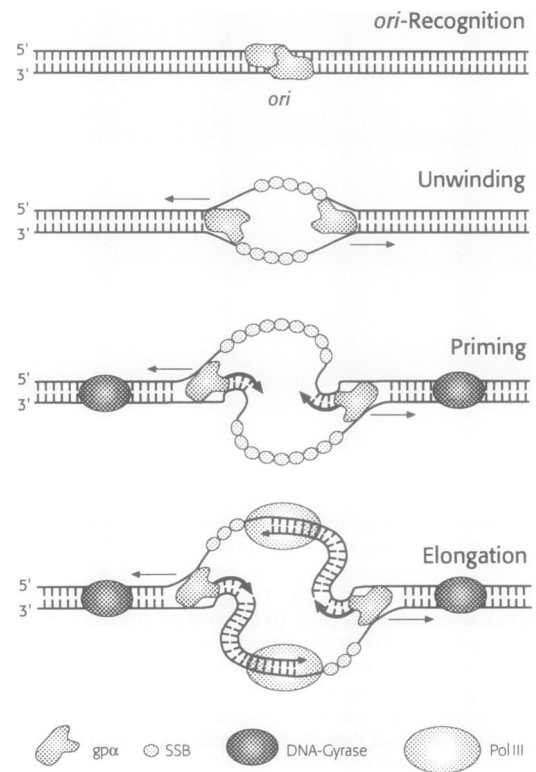


Figure 5. Model for P4 DNA replication initiation. See the Discussion section for details.

replication of P4 *in vitro*. The priming enzymes DnaG and RNA polymerase and the replicative DNA helicase DnaB are dispensable. The assembly of an active P4 replisome, that means a complex of P4 DNA, gp α , DNA polymerase III holoenzyme, and SSB does not depend on the presence of chaperones DnaJ and DnaK.

DISCUSSION

In this paper, we present data obtained from an *in vitro* replication system demonstrating that phage P4 DNA synthesis (i) requires the phage-encoded multifunctional gp α , (ii) depends on the host-encoded proteins DNA gyrase, SSB, and DNA polymerase III holoenzyme, (iii) is independent of host-encoded initiation and priming proteins (DnaB, DnaG, RNA polymerase) and (iv) that the mode of replication is θ -type bidirectional via intermediates composed of double-stranded DNA. Some of our results on the *in vitro* P4 replication do not match results reported previously (7, 5). The reasons for this could be (i) the use of a different strain and differences in preparing the extract required for the assay, (ii) different assay conditions, and (iii) differences in generating replicative intermediates. In the study mentioned, it was postulated that the frequency of lagging strand initiations was low, since *in vivo* replicating intermediates exhibited large single-stranded regions up to 6 kb (5). In our *in vitro* system, only a few small segments (≤ 0.4 kb) observed at the replication forks were single-stranded, indicating that lagging strand synthesis is initiated frequently after gp α has entered the duplex. Our results suggested that both daughter strands, once initiated at *ori*, are

synthesized continuously in the 5' → 3' direction until the site of replication termination is reached. Leading and lagging strand synthesis probably may occur concurrently at both forks. Host proteins DnaB and DnaG do not seem to influence neither lagging strand initiation nor elongation, since we could not detect any differences between intermediates obtained in the presence or absence of DnaB- or DnaG-specific antisera.

Like phage P4, plasmid RSF1010 replicates independently of host-encoded initiation and priming proteins (23). The plasmid's replicative helicase RepA unwinds DNA in 5' → 3' direction with respect to the strand it has bound. In contrast, gpα has 3' → 5' polarity which is unique among essential prokaryotic replicative helicases. The structure of *in vitro* replicating DNA molecules of the mammalian virus SV40 resembles that of P4 intermediates (24). The well-characterized large T antigen, which is the only virus-encoded protein essential for SV40 replication, combines two replication activities on a single polypeptide chain: origin recognition and 3' → 5' helicase (25, 26, 27). Therefore, the replication mechanisms of P4 and SV40 may share similarities in the initiation and elongation stages four major steps (24): (i) recognition and binding to the origin; (ii) local unwinding of the *ori* region; (iii) initiation of DNA synthesis by formation of RNA primers; (iv) elongation of nascent chains (Fig. 5), and to complete the round of replication, termination and maturation of the daughter molecules.

Formation of a gpα-*ori* complex seems to be complex. Several gpα molecules probably are involved, since footprinting analysis demonstrated (3), that gpα binds to all six type I repeats (TGTTCCACC) located within 104 bp of the *ori* region (P4 pos. 9313–9416, ref. 28). Large T antigen assembles as a double hexamer at the SV40 origin (29). It is conceivable that gpα also forms a nucleoprotein complex containing several gpα molecules. The P4 origin includes two segments of a markedly high AT-content (≥ 70%) within the type I iteron domain and another one comprising the three type II repeats, which are located at P4 pos. 9179–9208 (right and left part of *ori*, respectively; see Fig. 4). At one of these segments, the duplex might be locally melted, as generally observed for AT-rich, iteron-containing segments in other replication origins (30). Since the smallest replication bubbles were oriented towards the left part of *ori* (Fig. 4), we speculate that initial unwinding might occur at the type II repeats. Then, gpα separates duplex DNA by the intrinsic helicase activity fueled by hydrolysis of (d)NTPs, translocating in the 3' → 5' direction on the template for the leading strand (Fig. 5).

The absolute requirement for SSB in P4 replication indicates that its role is not only to stabilize the unwound strands, but it might also function in the formation of a nucleoprotein complex capable of unwinding and/or priming at a replication fork in its nascent state. SSB may also physically interact with gpα. The requirement for SSB reflects the situation in SV40 replication, where RP-A, a single-stranded DNA binding protein of the host, is essential (31).

The formation of the 'P4 primosome' diverges from most other known prokaryotic systems, because it probably includes the involvement of the essential *cis*-acting replication element (*crr*) *in vivo* (4). When all components of the replication assay have been mixed, a time lag was observed until DNA synthesis started (Fig. 1). This may be due to a rather slow assembly process which might be the most time consuming step in P4 DNA replication. Initiation complex formation generally is rate limiting also in other systems. In the presence of P4 form I DNA and gpα, a looped

structure was visible in the electron microscope (3), but the replicative intermediates did not exhibit looping or higher-order structures. A complex connecting *ori* and *crr* via gpα has not been detected even in a small portion of replicating molecules. We believe that *crr* plays a role rather in initiation and/or priming than in elongation. Thus, the actual function of *crr* needs further attention. *In vivo* data indicate, that DnaA protein is dispensable for P4 replication (6). However, it is interesting, whether the DnaA-binding consensus sequence in *crr* (TTATCCACA, ref. 32; P4 pos. 4284–4292) has any functional relevance.

Our system can be used to study in detail the effects of mutant gpα polypeptides on replication. For this purpose, we generated primase-null and helicase-null point mutations. Complementation analysis of a helicase- with a primase deficient gpα or *vice versa* will allow us to address the question, whether several distinct gpα molecules are responsible for the different stages of initiation/priming/elongation or whether one gpα complex assembled at *ori* primes and drives the fork ahead. Furthermore, a new approach to analyze interactions between gpα and components of host replication machinery will be opened, since preliminary data indicate that gpα stimulates ColE1 replication most probably during fork movement.

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