Two-dimensional gel analysis of rolling circle replication in the presence and absence of bacteriophage T4 primase

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ABSTRACT

The rolling circle DNA replication structures generated by the *in vitro* phage T4 replication system were analyzed using two-dimensional agarose gels. Replication structures were generated in the presence or absence of T4 primase (gp61), permitting the analysis of replication forks with either duplex or single-stranded tails. A characteristic arc shape was visualized when forks with single-stranded tails were cleaved by a restriction enzyme with the help of an oligonucleotide that anneals to restriction sites in the single-stranded tail. After calibrating the gel system with this well-studied rolling circle replication reaction, we then analyzed the *in vivo* replication directed by a T4 replication origin cloned within a plasmid. DNA samples were generated from infections with either wild-type or primase-deletion mutant phage. The only replicative arc that could be detected in the wild-type sample corresponded to duplex Y forms, consistent with very efficient lagging strand synthesis. Surprisingly, we obtained evidence for both duplex and single-stranded DNA tails in the samples from the primase-deficient infection. We conclude that a relatively inefficient mechanism primes lagging strand DNA synthesis *in vivo* when gp61 is absent.

INTRODUCTION

Bacteriophage T4 is extremely useful for studying the mechanism of DNA replication, in part because the phage encodes all of its own replication proteins in a relatively small genome. These proteins have been purified and used to establish replication forks on artificially primed templates (for a review see 1). The *in vitro* reaction depends on the phage-encoded DNA polymerase (gene product 43, gp43), polymerase accessory proteins (gp44/62 and gp45), single-stranded DNA (ssDNA) binding protein (gp32), helicase–primase complex (gp41/61) and helicase assembly factor (gp59). gp45 acts as a sliding clamp, allowing the

polymerase to synthesize DNA in a highly processive manner (2–4). The ssDNA binding protein is important for T4 DNA replication, recombination and repair (for reviews see 1,5). In the *in vitro* replication system, gp32 coats ssDNA and is thought to play important roles in both template unwinding and coordination of primer synthesis on the lagging strand. The replicative helicase, gp41, unwinds the parental helix ahead of the leading strand polymerase by tracking along the lagging strand template (6). In association with gp41, the primase (gp61) synthesizes pentaribonucleotide primers for Okazaki fragment synthesis on the lagging strand (7–10).

Within the infected cell, T4 DNA replication initiates by two different strategies (for a review see 11). At early times of infection, replication is initiated from any of several origins scattered throughout the genome. As the infection progresses, these origins are shut off and a recombination-dependent mode of replication becomes dominant. The recombination-dependent mode is believed to involve invasion of a duplex phage chromosome by a single-stranded genomic 3'-end, followed by assembly of a replication complex in the resulting D loop and use of the invading 3'-end as a primer for leading strand synthesis.

Two T4 replication origins, *ori(34)* and *ori(uvsY)*, are able to direct autonomous replication of pBR322 derivatives into which they are cloned (12,13). Replication of such T4 origin-containing plasmids begins shortly after phage infection and requires T4 DNA polymerase and the polymerase accessory proteins (14). Somewhat surprisingly, a deletion of the T4 primase gene does not eliminate either T4 origin-containing plasmid DNA replication or phage DNA replication, although in both cases replication is severely delayed (14; see also 15–18).

The DNA delay phenotype of T4 primase mutants has two interesting features. First, replication is quite deficient at early times of infection, arguing that primase is normally a key component of the replication machinery. Lagging strand synthesis would be expected to require primase and the enzyme could also be necessary to prime leading strand synthesis from T4 origins at early times. Second, replication eventually reaches a vigorous rate despite the absence of the T4-encoded primase. Although it has not been proven, it seems likely that leading

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strand replication from the origins is primed by an origin transcript at late times during primase-deficient infections. Several models can be considered for the generation of duplex replication products in the absence of primase: (i) priming of lagging strand replication by an alternative DNA primase; (ii) cleavage of recombinational junctions by endonuclease VII (gp49) to provide primers for subsequent replication (19); (iii) priming of the lagging strand by RNA polymerase-generated transcripts; (iv) annealing of single-stranded products of oppositely oriented replication forks.

Both *in vivo* DNA replication and the *in vitro* replication system can use plasmid templates to produce concatemeric products through a rolling circle mechanism of replication (20–23). In the *in vitro* system, replication is initiated artificially by providing a strand-specific nick. The 3'-hydroxyl at the nick is extended by the DNA polymerase holoenzyme complex to replicate the DNA on the leading strand, while lagging strand synthesis using the displaced strand as template depends on periodic RNA priming by the helicase-primase complex. The generation of rolling circles from the origin-containing plasmid *in vivo* is not understood. Replication probably initiates in a bidirectional mode to generate a θ form, which is then converted into a rolling circle, perhaps by breakage of the leading strand template at one of the two forks.

In order to address the role of T4 primase *in vivo*, the two-dimensional agarose gel method of Brewer and Fangman (24) was utilized to examine replicative structures in primase-proficient and primase-deficient infections. Because the arcs generated in these two-dimensional gels by rolling circle intermediates have not been well characterized, we first calibrated the system using the products of *in vitro* reactions with and without primase.

MATERIALS AND METHODS

Materials

Restriction enzymes, random primed labeling kit, [α - 32 P]dATP and oligonucleotides were purchased from commercial sources. Oligonucleotides were also synthesized by the Duke University Botany Department Oligonucleotide Synthesis Facility. The T4 origin-containing plasmid, pGJB1, has been described elsewhere (25,26). L Broth contains NaCl (10 g/l), BactoTryptone (10 g/l) and yeast extract (5 g/l) and was supplemented with ampicillin (25 mg/l) for growth of plasmid-containing cells.

Strains

The non-suppressing *Escherichia coli* host strain AB1 [*araD139* Δ (*ara-leu*)7697 Δ *lacX74 galU galK hsdR rpsL*] was described by Kreuzer *et al.* (13). T4 strain K10, which is considered the wild-type control in these experiments, has the following mutations: *ambB262* (gene 38), *amS29* (gene 51), *nd28* (*denA*) and *rIIPT8* (*rII-denB* deletion) (27). K10-61 Δ is isogenic except that it harbors an extensive in-frame deletion of gene 61 (14).

In vitro replication assay

Double-stranded M13mp19 DNA was incubated with phage fd gene 2 protein to produce a uniquely nicked substrate as previously described (28,29). The gene 2 protein nicking reaction was ~80% complete, explaining the presence of intact monomeric

topoisomers in the two-dimensional gels. A small percentage (~1–5%) of the substrate contained randomly located nicks. The *in vitro* replication reactions contained 0.15 μ g M13mp19 DNA that had been nicked as described above, 33 mM Tris-acetate, pH 7.8, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol, 1 mM GTP, 150 μ M of each of the other seven deoxyribo- and ribonucleotide triphosphates and the following T4 replication proteins: 20 μ g/ml helicase (gp41), 100 μ g/ml ssDNA binding protein (gp32), 1 μ g/ml DNA polymerase (gp43), 25 and 11 μ g/ml polymerase accessory proteins (gp44/62 and gp45, respectively), 0.5 μ g/ml helicase-primase loading protein (gp59) and, where indicated, 1.5 μ g/ml primase (gp61). After incubation at 37°C for 8.5 min, 35 mM Na₃EDTA and 0.1% SDS were added to stop DNA synthesis and the reaction products were stored at –70°C prior to gel analysis.

Alkaline agarose gel electrophoresis

Alkaline agarose gel electrophoresis was performed as described by Formosa and Alberts (30) with the following variations. Samples were denatured by bringing them to 300 mM NaOH and a 1/5 vol. stop solution (5% SDS, 15% Ficoll, 0.25% bromophenol blue and xylene cyanol FF) was then added. The denatured samples were loaded onto a 0.5% agarose gel (13 $\times$ 20 cm) which was subjected to electrophoresis in a solution of 30 mM NaOH and 2 mM Na₃EDTA with recirculation for 20 h at 30 V. Gels were neutralized in 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5, prior to Southern blotting as described by Sambrook *et al.* (31) and the blots were probed with labeled DNA (Boehringer Mannheim Random Primed Kit) prepared from an M13 ssDNA template (from US Biochemical).

Two-dimensional gel analysis of *in vitro* samples

Some DNA samples were loaded directly onto the first dimension agarose gel; others were treated with proteinase K at 37°C for 15 min, extracted once with phenol, passed through a CL6B spin column and digested with *Alw*NI for 1 h at 37°C in the presence or absence of 90 pmol oligonucleotide, 5'-GCGCAGTCTCT-GAAT-3', which anneals to the *Alw*NI site in M13 plus strand DNA (the displaced strand that normally serves as lagging strand template). The conditions for two-dimensional gel electrophoresis were based on those described by Brewer and Fangman (24). Briefly, the first dimension gel contained 0.4% agarose and was run in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM Na₃EDTA) at 1 V/cm for 48 h. The second dimension gel contained 1.0% agarose and ethidium bromide (0.3 μ g/ml) and was run in TBE buffer containing ethidium bromide (0.3 μ g/ml) for 22 h at 6 V/cm at 4°C with buffer recirculation. The two-dimensional gels were analyzed by Southern hybridization (31) and the blots were probed with labeled DNA (Boehringer Mannheim Random Primed Kit) prepared from an M13 ssDNA template (from US Biochemical).

In vivo plasmid replication

Plasmid replication assays were performed essentially as described by Kreuzer and Benson (14). *Escherichia coli* AB1 cells containing plasmid pGJB1 or pBR322 (as indicated) were grown to a density of 4×10^8 cells/ml with vigorous shaking at 37°C in L broth. Where indicated, the cells were infected with either T4 K10 or K10-61 Δ at a multiplicity of 3 plaque-forming units/cell.

After a 3 min attachment period without shaking, the infected cells were incubated at 37°C with vigorous shaking for 20 min. DNA was isolated by pelleting the cells in a microcentrifuge, resuspending the pellet in lysis buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.2% SDS, 10 mM Na₃EDTA, 0.3 mg/ml proteinase K) and incubating at 37°C for 10 min. The lysates were then placed in a microtiter plate and trioxsalen (Sigma Chemical Co.) was added to each sample (final concentration 2 µg/ml). The DNA was cross-linked by illuminating with long wavelength UV light at 4 cm for 10 min (4.5 mW/cm²). The samples were incubated at 65°C for 1 h, sequentially extracted with phenol, phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol and finally dialyzed against TE (10 mM Tris-HCl, pH 7.8, 0.5 mM Na₃EDTA) at 4°C overnight. The DNA samples were stored at -70°C prior to gel electrophoresis.

Two-dimensional gel analysis of *in vivo* samples

The DNA samples were digested with *Dra*I for 2 h at 37°C. When samples were digested in the presence of oligonucleotides to render ssDNA susceptible to restriction enzyme cleavage, 110 pmol of the oligonucleotide *Dra*A (5'-TTGATTTAAACTTCATTTTAAATTTAAAGGA-3') was added after the initial 2 h digest and the samples were heated to 65°C and then allowed to cool slowly to 37°C. The *Dra*A oligonucleotide hybridizes to one strand of pBR322-derived plasmids in the interval 3228-3260 (pBR322 map coordinates), thereby rendering two potential *Dra*I sites double helical. After this annealing, the digest was supplemented with additional enzyme and incubated at 37°C for another 2 h. The annealing step was then repeated after the addition of 220 pmol of the second oligonucleotide, *Dra*B (5'-TCCTTTTAAATTAAAAATGAAGTTTAAATCAA-3'), which hybridizes to the same region of pBR322 but on the opposite strand. The heating, cooling and additional enzyme additions were also performed for those samples that were digested in the absence of oligonucleotide, so that the products would be directly comparable. The conditions for two-dimensional gel electrophoresis were identical to those described above, except that the first dimension gel was run for only 24 h (because the monomer DNA length is shorter). The two-dimensional gels were analyzed by Southern hybridization (31) and the blots were probed with randomly labeled DNA (Boehringer Mannheim Random Primed Kit) prepared from a denatured *Ase*I-linearized pBR322 DNA template.

RESULTS

Analysis of *in vitro* replication structures

The two-dimensional agarose gel electrophoresis system of Brewer and Fangman (24) provides a powerful technique for analyzing the structure of DNA replication intermediates because it allows separation of molecules according to both mass (first and second dimensions) and shape (second dimension). We wished to use this system to analyze the structure of plasmid replication intermediates that form when phage T4 infects cells that carry a T4 origin-containing plasmid. However, these intermediates were believed to be rolling circles (20,21) and relatively little information was available on the patterns generated in two-dimensional gels by rolling circles. Therefore, we began by using the well-characterized T4 *in vitro* replication system to calibrate the system. When provided with a nicked circular substrate, the

Figure 1. Analysis of *in vitro* replication products by alkaline agarose gel electrophoresis. Uncleaved *in vitro* replication reaction samples (+gp61, top and -gp61, bottom) were denatured and subjected to electrophoresis through an alkaline 0.5% agarose gel. The DNA was visualized by Southern blotting with an M13 minus strand probe. Direct radioisotope counting was performed using an AMBIS imager. The peak labeled 'nicked' consists of unreplicated substrate plus strand (nicked by the gene 2 protein), while the peak labeled 'unnicked' consists of covalently closed interlocked ssDNA circles that result from denaturing the substrate molecules that escaped gene 2 nicking. Size markers (denatured λ DNA and λ *Hind*III fragments, in kb) are noted along the bottom of the profile.

in vitro T4 replication system generates rolling circles by chain extension from the nick (22,23). For a template, we used double-stranded M13mp19 DNA with a site-specific nick introduced by the fd gene 2 protein. The reactions were performed either in the presence or absence of the T4-encoded primase (gp61) in order to generate rolling circles with either single-stranded or duplex concatemeric tails.

The expected reaction products were first verified by one-dimensional alkaline agarose gel electrophoresis. Quantitation of a Southern blot of the gel revealed the expected reaction products, along with remaining substrate DNA (Fig. 1). The profiles show products longer than 50 kb in both reactions, consistent with the expected leading strand of rolling circles (substrate monomer length 7.25 kb). In addition, short products consistent with Okazaki fragments were generated only in the primase-containing reaction. The two peaks labeled 'nicked' and 'unnicked' comprise substrate DNA (see Fig. 1 legend). With verification of the expected products of rolling circle replication, we turn to the two-dimensional gel analysis.

To help explain the two-dimensional gel results below, Figure 2 shows the replicative forms expected with either efficient, delayed or no lagging strand synthesis (Fig. 2A-C respectively). N represents the site of the nick introduced by fd gene 2 protein and filled triangles represent *Alw*NI cleavage sites that have not been cleaved. The left panel presents molecules prior to restriction enzyme cleavage, while the right panel shows the same molecules after cutting with *Alw*NI.

Figure 3 shows typical two-dimensional gel patterns generated from the *in vitro* reaction products, visualized by Southern hybridization with an M13 probe. The samples in Figure 3A (+ primase) and B (- primase) were not digested with restriction enzyme prior to electrophoresis. In both electropherograms, a ladder of intact supercoiled *topoisomers* and a spot of *nicked circles*, presumably consisting of unreplicated substrate, are

Figure 2. Diagram of rolling circle forms both before and after restriction enzyme cleavage. The first and later rounds of rolling circle replication are depicted, with and without lagging strand synthesis (A and C). A molecule with a partially completed lagging strand is also shown (B). The nicking site used to initiate replication is indicated by the letter N and uncleaved *Alw*NI sites are indicated by filled triangles. The dashed lines at the end of concatemer tails indicate that the tails may be much longer than represented in the diagram.

clearly visible (Fig. 3A and B). These and additional forms below were assigned based in part on their migration compared with molecular weight markers in both dimensions (data not shown).

In addition, a *ds linear arc* is particularly prominent in the products generated with primase (Fig. 3A). This *ds linear arc* very likely consists of molecules that were generated by 'onion skin' synthesis, as previously analyzed by Barry and Alberts (32). This type of replication, diagrammed in Figure 4A, occurs when an Okazaki fragment becomes displaced from the lagging strand, either by improper extension of the subsequent Okazaki fragment or by assembly of a new replication complex at the junction between two Okazaki fragments. In either case, the displaced Okazaki fragment is then converted into a duplex form by the

excess primase and DNA polymerase holoenzyme activities in the reaction.

DNA from the primase-containing reaction (Fig. 3A) generated a characteristic curve, the *ds eyebrow*, emanating from the nicked circle spot. [The name *ds eyebrow* was used by Preiser *et al.* (33) for a similar curve from malarial mitochondrial DNA]. We are certain that this *ds eyebrow* curve consists of rolling circles with duplex tails (see Fig. 2A, left panel) for the following reasons: (i) the curve begins at the position of the nicked circle and increases in mass as expected for rolling circles with increasingly long tails; (ii) previous studies (22,23,34,35) have demonstrated rolling circle replication in this system; (iii) the curve is dependent on the presence of primase; (iv) restriction enzyme treatment apparently converts the curve into a simple Y arc (see below).

The *ds eyebrow* curve disappeared when the samples were prepared without primase (Fig. 3B). In this case, priming cannot occur on the lagging strand and the concatemer tail should thus remain single-stranded. In place of the *ds eyebrow*, a continuously upward-moving arc emanates from the nicked circle spot. The presence of a single-stranded tail apparently causes the rolling circles to run differently through the gel, producing a characteristic curve which we call the *ss eyebrow* (see Fig. 2C, left panel).

The gels shown in Figure 3C and D display the results obtained when the same two DNA samples were cleaved prior to electrophoresis with *Alw*NI, which cuts double-stranded M13 DNA once (~50% of the M13 DNA length away from the nicking site), but is unable to cleave M13 plus strand ssDNA. When primase had been present in the *in vitro* reaction, a standard *duplex Y arc* was generated (Fig. 3C). The *duplex Y arc* begins at the *ds monomer* spot and returns to the *ds linear arc* at $2 \times$ M13 size, behaving exactly as expected for cleaved rolling circles with a completely replicated concatemer tail (see Fig. 2A, later rounds). An additional Y arc is observed beginning at the *ds monomer* spot and returning to the *ds linear arc* at $\sim 1.5 \times$ M13 size. This additional Y arc (*first round Y arc*) is expected to be generated from molecules that were in the first round of replication, since the nicking site used to initiate replication, rather than an *Alw*NI site, forms the end of the concatemer tail and thus one end of the Y molecule (see Fig. 2A, first round). An unlabeled arc is faintly visible in Figure 3C, beginning near the end of the *first round Y arc* and staying beneath the *duplex Y arc*. We infer that this faint arc consists of first round molecules in which replication has passed the *Alw*NI site, but the site has not yet become duplex (which is necessary for *Alw*NI cleavage; see below).

Several notable features are obvious with the *Alw*NI-cleaved reaction products that had been generated without primase (Fig. 3D). First, a prominent arc of long ssDNA linear molecules is evident (*ss linear arc*). Analysis by one-dimensional alkaline agarose gels indicated that purification of the DNA prior to restriction enzyme treatment caused a low level of random breakage of the single-stranded rolling circle tails (data not shown). Second, a long continuous arc emanates from the *ds monomer* (linear) spot and continues diagonally up the gel (Fig. 3D, denoted *uncut ss tails*). This long arc must have been generated from rolling circles with single-stranded tails, with *Alw*NI cleaving the duplex template circle but not the single-stranded concatemer tail (see Fig. 2C, first round, cut; later rounds, cut, no oligo). Third, a distinct arc begins at the same *ds monomer* site but hooks over at a relatively small size. This *first*

Figure 3. Two-dimensional gel analysis of *in vitro* samples. The conditions for the *in vitro* reactions and for the two-dimensional gel electrophoresis are described in Materials and Methods. The first dimension is represented horizontally (left to right) and the second dimension vertically (top to bottom). The DNA samples for (A), (C) and (E) were produced in the presence of T4 gp61, whereas those for (B), (D) and (F) were generated without gp61. The samples in (A) and (B) were not cleaved with restriction enzyme, while those in panels (C)–(F) were cleaved with *Alw*NI; an oligonucleotide complementary to the *Alw*NI site was added to the digests for (E) and (F). Each panel is a Southern blot of the two-dimensional gel, using randomly labeled M13 minus strand DNA as probe.

Figure 4. Diagram of replicative structures generated from 'onion skin' synthesis (A) and strand switching (B) mechanisms. During 'onion skin' synthesis, displacement of an Okazaki fragment and subsequent replication of the fragment into a duplex results in short, linear duplex molecules. The strand switching mechanism invokes template strand switching by the T4 DNA polymerase, leading to the production of duplex concatemer tails (and duplex Y forms) even in the absence of primase.

round Y arc is expected from first round replication structures that contain a discrete end on their concatemer tail (the nicking site; molecules represented in Fig. 2C, first round, cut).

We wished to further analyze the branched DNA forms that contain single-stranded regions by adding an oligonucleotide complementary to the restriction enzyme site during the *Alw*NI digest. Control experiments demonstrated that *Alw*NI is unable to cleave M13 plus strand ssDNA, but annealing of the appropriate oligonucleotide allows efficient cleavage (Fig. 5, compare lanes 1, 3 and 5). Furthermore, *Alw*NI cleavage of the ssDNA in the presence of oligonucleotides is highly site specific, as shown by the production of two appropriate discrete fragments in a double digest with *Alw*NI and *Hind*III (Fig. 5, lane 7; oligonucleotide for *Hind*III site was also present).

When the oligonucleotide was added to the digestion of the *in vitro* replication products produced with primase, two differences emerged (Fig. 3E; compare with Fig. 3C). First, an intense new arc (*monomer ss/ds arc*) was generated, beginning at the *ds monomer* (linear) spot and ending at the *ss monomer* (linear) spot (which ran off this particular gel). The molecules within this arc must be partially duplex, monomer length fragments with one *Alw*NI end in a single-stranded form. We presume that this *monomer ss/ds arc* originates from partially primed concatemer tails, for example regions where the 5'-end of the most recent Okazaki fragment is relatively distant from the fork (in Fig. 2B, right panel, the portion of the concatemer tail that would be released when oligonucleotide is added to allow *Alw*NI cleavage of ssDNA; see also Discussion for a description of other ss/ds monomer fragments). The second notable difference generated by the addition of oligonucleotide is the generation of a (faint) novel Y arc, referred to as the *oligo-dependent arc* (Fig. 3E). This arc appears to start at the *ds monomer* spot and presumably

Figure 5. Specific cleavage of single-stranded M13 DNA by *AlwNI* and *HindIII* aided by oligonucleotides. All reactions contained 0.4 μ g M13 ssDNA. In addition, the reactions contained: *HindIII* (lane 2); *AlwNI* (lane 3); *HindIII* plus an oligonucleotide for the *HindIII* site (lane 4); *AlwNI* plus an oligonucleotide for the *AlwNI* site (lane 5); *HindIII*, *AlwNI* plus an oligonucleotide to the *HindIII* site (lane 6); *HindIII*, *AlwNI* plus oligonucleotides to both the *HindIII* and *AlwNI* sites (lane 7). Samples were loaded onto a 1.0% agarose gel in TBE and electrophoresed for 18 h at 25 V. Circular and linear M13 ssDNA forms are noted with arrows. A molecular weight scale generated from the migration of double-stranded size markers (in kb) is indicated to the left of the gel.

contains molecules with concatemer tails that are single stranded, at least up to the first *AlwNI* site (see Fig. 2B, cut, plus oligo; see also below). The darkened broad area between this *oligo-dependent arc* and the *duplex Y arc* presumably contains Y forms with partial duplex tails.

When oligonucleotide was added to the sample prepared without primase (Fig. 3F), the long arc denoted *uncut ss tails* (Fig. 3D) disappeared due to *AlwNI* cleavage of the ssDNA tails. In its place, an intense *oligo-dependent arc* was generated. This arc, which begins at the *ds monomer* spot, must contain Y-shaped molecules with one ssDNA arm, as expected from rolling circles without lagging strand synthesis (see Fig. 2C, later rounds, cut, plus oligo). In addition, the DNA that formed the *ss linear arc* in Figure 1D was digested in the presence of oligonucleotide and most of the resulting molecules presumably migrated at the position of the *ss monomer* spot (much of this spot ran off the gel shown in Figure 3F, but repeats with shorter electrophoresis times showed an intense *ss monomer* spot). A very faint *duplex Y arc* was detected in both cleaved samples from the primase-deficient reactions, indicating that a replication fork occasionally had a double-stranded concatemer tail even in the absence of primase (Fig. 3D and F; the arc contains discrete spots and is not labeled). We surmise that these molecules result from strand switching by DNA polymerase, followed by replication of the single-stranded tail (see Fig. 4B and Discussion).

From these *in vitro* studies we were able to characterize a variety of unique forms associated with rolling circle replication. In particular, the addition of appropriate oligonucleotides during restriction enzyme digestion allows the clear visualization of single-stranded replicative structures. Thus informed, we turned our attention to the characterization of the replication structures found *in vivo*.

Analysis of *in vivo* replicated plasmid DNA

Either of two T4 origins, *ori(34)* or *ori(uvsY)*, can direct autonomous replication of a pBR322 plasmid upon bacteriophage T4 infection.

The origin plasmid likely replicates, at least in part, by a rolling circle mechanism, because long concatemeric DNA products can be visualized by electron microscopy or by pulsed field gel electrophoresis (20,21). We began the *in vivo* experiments by comparing the replicative forms of pGJB1, an *ori(uvsY)*-containing plasmid, to those generated by a pBR322 control plasmid. DNA samples were prepared 20 min after infection of the plasmid-bearing *E. coli* cells by a T4 mutant deficient in host DNA breakdown. A DNA sample from the pGJB1-containing cells was also analyzed without T4 infection to determine whether the branched DNA forms are dependent upon phage infection. The DNA samples were cross-linked with trioxsalen immediately after cell lysis to reduce the possibility of DNA branch migration and then the samples were treated with the restriction enzyme *DraI* and subjected to two-dimensional gel electrophoresis.

In the absence of T4 infection, the pGJB1 plasmid generated a faint arc, presumably due to replication triggered from the *ColEI* origin (Fig. 6A). This *theta* arc is exactly as predicted from the analysis of pBR322 replicative forms by Martin-Parras *et al.* (36). Furthermore, the results of Martin-Parras *et al.* (36) predict that the *theta* arc should give way to a single-Y arc and then a late stage double-Y form termination intermediate upon *DraI* digestion; these are very likely the spots within the region labeled *Y forms* in Figure 6A. Note that a simple *duplex Y arc* was not detected in this control sample without T4 infection.

Turning to the analysis of samples from T4-infected cells (Fig. 6B and C), we need to first explain the cytosine modifications introduced during T4 replication and their effect on restriction enzyme digestion. T4 replicated DNA contains glucosylated hydroxymethylcytosine residues in place of normal cytosine, making the DNA refractory to most restriction enzymes. *DraI*, which cleaves a hexameric sequence with only AT base pairs, is one of the few restriction enzymes capable of cleaving modified T4 DNA. Nonetheless, *DraI* cleaves T4-modified (replicated) and unmodified (unreplicated) plasmid DNA differently. This differential cleavage is the cause of the two discrete *ds monomer* spots that are visible along the *ds linear arc* in Figure 6B (and in the gels below). Plasmid pGJB1 actually has three *DraI* sites, but two of them are only 19 bp apart and therefore behave as a single site in this study. The third site is resistant to *DraI* cleavage only when the substrate is modified, presumably because modified cytosines adjacent to this site block the restriction enzyme. Therefore, modified (replicated) pGJB1 is cleaved only once with *DraI*, generating the larger (4.48 kb) band, while unmodified (unreplicated) plasmid is cut at both sites, generating the smaller (3.79 kb) band (and a 0.69 kb band that runs off these gels).

Figure 6B displays a typical result from a T4 infection of cells harboring the *ori(uvsY)* plasmid. We detected a strong *duplex Y arc* similar to that generated in the *in vitro* reactions, consistent with rolling circle replication. In addition, we detected a clear *X arc*, presumably containing plasmid recombination intermediates. Note that the *duplex Y arc* originates only from the modified DNA spot, implying that virtually all Y form DNA consists of replicated plasmid DNA (see also below). Importantly, no *duplex Y arc* was detected when T4 infected cells with the control plasmid that lacks *ori(uvsY)* (Fig. 6C). The *Y forms* generated with the T4 origin plasmid are therefore dependent on the presence of the T4 origin of replication as well as on T4 infection.

Essentially identical *duplex Y arcs* were detected after cleaving replicated pGJB1 DNA with restriction enzymes that cut (modified DNA) at other locations in the plasmid (data not

Figure 6. Two-dimensional gel analysis of plasmid replication with and without T4 infection. DNA was prepared from uninfected cells containing the T4 origin plasmid pGJB1 (A), from a T4 (gene *6I*⁺) infection of cells containing pGJB1 (B), or from a T4 (gene *6I*⁺) infection of cells containing pBR322 (C). All samples were treated with restriction enzyme *Dra*I. Each panel is a Southern blot of the two-dimensional gel, using randomly labeled pBR322 DNA as a probe. Cytosine-containing (unmodified) monomer DNA is denoted *dC*, while the monomer DNA that contains glucosylated hydroxymethylcytosine (modified) residues is indicated by *dC**. The unlabeled monomer spot in (A) that is slightly larger than *ds monomer (dC)* represents unmodified DNA that has been partially cleaved by *Dra*I. There are three unlabeled monomer spots in (C), representing (from high to low molecular weight) modified monomer DNA, hemi-modified monomer DNA and partially cleaved unmodified DNA. The first two of these spots result from a very inefficient replication of pBR322 in T4-infected cells and they migrate more slowly than partially cleaved unmodified DNA because of the glucosyl modifications.

shown). This result demonstrates that the Y forms originate from σ -shaped (rolling circle) molecules rather than uni- or bidirectional θ forms. Indeed, we have been unable to detect arcs consistent with θ form plasmid replication from T4-infected cells (data not shown). The *ds eyebrow* form, previously characterized with uncut rolling circle DNA *in vitro*, was also detected with uncut *in vivo* samples of origin-containing plasmid DNA (data not shown; the *ds eyebrow* was not nearly as pronounced as in the *in vitro* samples, presumably due to a low abundance of rolling circles with short tails). We conclude that the major branched plasmid DNA form from wild-type infections is the *duplex Y arc*, very likely generated by rolling circle replication with efficient lagging strand synthesis (see Discussion).

As described in the Introduction, we are interested in the nature of replication that occurs in a primase-deficient (gene *6I* deletion mutant) infection. We therefore modified the oligonucleotide system for cleaving ssDNA so that it could be used with *in vivo* plasmid DNA samples. Unlike *in vitro* replication, we could not predict which strand (if either) of the origin-containing plasmid might remain single stranded in the absence of primase. Therefore, two oligonucleotides were used sequentially so that either DNA strand would be cleaved.

Figure 7 displays typical Southern blots of two-dimensional gels of pGJB1 plasmid DNA after infection by wild-type (Fig. 7A and B) or primase-deficient phage (Fig. 7C–E), without (A and C) or with added oligonucleotides (B, D and E). With wild-type phage, we again detected a *duplex Y arc* (whether or not the restriction digest contained oligonucleotide to allow ssDNA cleavage; Fig. 7A and B). Furthermore, the addition of oligonucleotide to allow cleavage of single-stranded forms caused no detectable changes in the two-dimensional pattern (compare Fig. 7A and B).

When we examined plasmid DNA from the infection with primase-deficient phage, the overall pattern was surprisingly similar to that of the wild-type (compare Fig. 7A and C). A *duplex Y arc* was readily detectable even without oligonucleotides that allow ssDNA cleavage (Fig. 7C). This result suggests that lagging strand synthesis occurred despite the absence of gp61 (see Discussion). In the digest without added oligonucleotides, DNA from the *6I* mutant infection produced a line (labeled *ss tails*) that appears to emanate from the *ds monomer* spot and move up into the area beneath the *duplex Y arc*. Based on the analysis of the *in vitro* samples above, this line behaves like Y structures with one single-stranded arm, consistent with rolling circles that do not contain a fully functional lagging strand complex.

As mentioned above, the addition of oligonucleotide did not change the pattern of the wild-type sample (compare Fig. 7A and B). Therefore, in a wild-type infection, single-stranded structures are rare or absent. However, in the sample from the primase-deficient phage infection, the addition of oligonucleotides noticeably altered the electropherogram (compare Fig. 7C with D and E; Fig. 7E displays a lighter exposure of the blot in Fig. 7D). First, the line labeled *ss tails* became more prominent, consistent with the assignment of the molecules in this line as replication forks with non-functional lagging strand complexes. Second, we detected a prominent arc beneath the *ds monomer* (Fig. 7D and E). As with the *in vitro* samples (Fig. 3E), a *monomer ss/ds arc* appears to connect monomer linear dsDNA and monomer linear ssDNA, strongly arguing that the arc contains partial-duplex linear fragments. This assignment is supported by the fact that this arc disappears when the DNA sample is denatured prior to electrophoresis (data not shown).

These results indicate that the presence of abundant ssDNA is unique to the primase-deficient mutant, but that the detectable

Figure 7. Two-dimensional gel analysis of plasmid replication by the T4 wild-type and a primase-deficient mutant. DNA was prepared from wild-type T4 infections of cells containing the T4 origin plasmid pGJB1 (A and B), or from primase-deficient mutant phage infections of the same plasmid-bearing host strain (C–E). All samples were treated with the restriction enzyme *DraI*. The restriction enzyme digests for (A) and (C) did not contain added oligonucleotide, whereas those for (B), (D) and (E) contained oligonucleotide to permit *DraI* cleavage of ssDNA. (E) A lighter exposure of (D). Each panel is a Southern blot of the two-dimensional gel, using randomly labeled pBR322 DNA as a probe.

single-stranded regions are often contiguous with duplex regions. As already discussed, an apparently normal *duplex Y arc* was readily visualized in DNA from the primase-deficient infection, arguing that some of the rolling circles have a functional lagging strand replication complex.

DISCUSSION

In this study, we analyzed the two-dimensional gel profiles associated with rolling circle replication and probed the mechanism of T4 DNA replication in the presence and absence of primase (gp61). Characteristic shapes, the *ss eyebrow* and the *ds eyebrow*, were observed when rolling circles had not been cleaved by restriction enzyme and the expected *duplex Y arcs* were observed after restriction enzyme treatment. In addition, the presence of an oligonucleotide during restriction enzyme treatment allowed unambiguous identification of structures with single-stranded regions. Our extensive characterization of various rolling circle structures and the method for cleaving single-stranded regions should be helpful to other workers analyzing unknown structures from a variety of systems.

We observed two features of the *in vitro* replication products that were not expected. First, the primase-containing reactions generated a very prominent arc of monomeric DNA that was partially single stranded when ssDNA cleavage was directed by the presence of the oligonucleotide. Given the fact that this arc is roughly as intense as the *ds monomer* spot, it seems unlikely that all of the DNA within this arc can be explained as regions of the lagging strand very close to the replication fork that were in the process of Okazaki fragment synthesis when the reaction was terminated. Most of the DNA in this *monomer ss/ds arc* probably originated from rolling circle tails in which the replication apparatus failed to complete one or more Okazaki fragments, thereby leaving ssDNA stretches in between adjacent Okazaki fragments. Such incomplete replication structures are commonly seen during electron microscopic analysis of T4 *in vitro* replication products (M.L.Wong and B.M.Alberts, unpublished data). A second unexpected feature was the presence of a faint *duplex Y arc* in the products of reactions lacking primase. This arc cannot result from gp61 contamination in one of the replication protein preparations, because all of the replication proteins were purified from overproducing clones. In addition, the arc consists primarily of a series of spots, rather than the more continuous line seen in the products of primase-containing reactions. The simplest explanation is that the T4 DNA polymerase undergoes strand switching (see Fig. 4B) at specific sites in the template circle, perhaps at inverted repeats where the newly synthesized 3'-end can hybridize with the ssDNA template tail (37–39). Once strand switching occurs, the site of the strand switch becomes a permanent Y junction and the tail of the rolling circle should be quickly replicated to completion.

Numerous investigators have used two-dimensional Brewer and Fangman (24) gels to characterize *in vivo* replication intermediates and the characterization of replication structures presented here should help in interpreting unusual arcs in both past and future studies. For example, Han and Stachow (40) observed an 'E-arc' from mitochondrial DNA of *Schizosaccharomyces pombe* which migrated much like our *ss tails*. Indeed, those authors provided evidence that the 'E-arc' contains DNA molecules with extensive single-stranded regions and argued that the arc consists of rolling circles with an unprimed tail. Our characterization of *bona fide* rolling circles with unprimed lagging strand tails supports the assignment made by Han and Stachow (40). In addition, Preiser *et al.* (33) recently detected *ds eyebrow* forms from uncut mitochondrial DNA of the malarial parasite *Plasmodium falciparum* and also concluded that these represent rolling circle intermediates.

The characterization of replicative structures from the *in vitro* system was designed to help decipher the plasmid DNA forms that are generated when phage T4 infects cells bearing a T4 origin-containing plasmid. The major branched DNA form detected from the primase-proficient infection was the *duplex Y arc*, as expected for rolling circle replication with normal lagging strand synthesis. Previous studies also suggested that T4 origin-containing plasmids use the rolling circle replication mode during phage infection, in that the major product of replication was found to be long concatemers of plasmid DNA (20,21).

Despite the strong evidence for rolling circle replication, we believe that replication from the T4 origin initiates with bidirectional θ forms which are converted to rolling circles at some frequency. The most direct evidence for this view comes from experiments in which a T4 origin was inserted into the *lac*

operon of the *E. coli* chromosome. The presence of the inserted origin increased replication of adjoining DNA during a T4 infection and all of the replicated DNA had undergone replication in both directions from the origin (unpublished data). Perhaps origin-containing plasmids initiate replication with a θ form which is then converted into a rolling circle at some frequency (analogous to replication of λ DNA; for a review see 41). Rolling circle intermediates should be much easier to detect because a rolling circle continues replication indefinitely. In addition, the products of rolling circle replication could predominate even if only a small fraction of θ forms are converted into rolling circles, because each rolling circle can generate numerous plasmid copies, whereas θ forms only duplicate the starting circle.

Several results argue that the *duplex Y arc* from the *in vivo* samples results from plasmid replication and not from recombination intermediates (e.g. by invasion of a linear end into an intact circle). First, the *duplex Y arc* was readily visualized from infections using a T4 mutant deficient in the recombination protein UvsY (synaptase accessory protein, required for UvsX-promoted recombination *in vivo*) (data not shown). Further, the modification state of the plasmid DNA argues strongly that the arcs are generated by replication. T4-directed modifications require *de novo* DNA synthesis, because the hydroxymethylcytosine is incorporated at the nucleotide level and glucosylation occurs subsequently on the hydroxymethyl residue. As shown in Figures 6B and 7, the arc emanates from the *DraI* monomer spot containing T4-modified DNA and not from the spot containing unmodified plasmid DNA. Furthermore, the addition of *HaeIII*, which cleaves unmodified (unreplicated) plasmid DNA into numerous small fragments, did not affect the shape or intensity of the *DraI*-generated arc (data not shown). Finally, no arc was detected when we used restriction enzymes that linearize unmodified plasmid but are unable to cleave T4-modified DNA (data not shown). We cannot rule out the possibility that the *duplex Y arc* originates via recombination between two plasmid DNA molecules that have already replicated. However, in this model, the recombination event would need to strongly prefer T4-modified DNA and would need to be independent of UvsY protein. It seems much more likely that the *duplex Y arcs* contain true replicative intermediates (or aborted replication products).

We are very interested in the mechanism of origin plasmid DNA replication after infection by the *6I* deletion mutant. A previous study showed that plasmid replication is deficient at early times but is quite robust by late times in the infection (14). In this study, we analyzed samples from a time (20 min) when plasmid replication is just beginning in the *6I* mutant infection; similar arcs were detected from a much later time point (data not shown). Two features of the two-dimensional gel patterns of DNA from the primase-deficient mutant are notable. First, the addition of oligonucleotide to the restriction enzyme reaction revealed the presence of ssDNA. Second, the *duplex Y arc*, indicative of double-stranded concatemer tails, was visualized even though T4 primase was absent.

The ssDNA detected in the primase-deficient infection consists of Y forms with ssDNA tails (presumably rolling circles with little or no lagging strand synthesis) and monomer length partial duplexes (presumably tails of rolling circle with partially replicated lagging strands). One interesting possibility which we are currently testing is that the rolling circles replicate in only one direction when primase is not present. Both *ori(uvsY)* and *ori(34)* contain a middle mode promoter which is necessary for function

of the origin (25,26). In several systems (ColE1, T7 and vertebrate mitochondria), a transcript made by RNA polymerase can act as a primer for leading strand DNA synthesis from the origin (42–46). It seems very likely that the RNA transcript from the T4 promoter within *ori(uvsY)* and *ori(34)* is used to initiate leading strand synthesis in the absence of primase. We do not know whether the origin transcript or a gp61-generated oligo-ribonucleotide primer is used to initiate leading strand synthesis in a primase-proficient infection (14).

The presence of a discrete *monomer ss/ds arc* in the primase-deficient infection suggests that some lagging strand synthesis occurs on the rolling circle tail, but that the mechanism is inefficient and leaves substantial single-stranded gaps. It is also interesting to note that these ssDNA forms were not detected from the primase-proficient infection, arguing for very efficient lagging strand synthesis when gp61 is available.

The presence of the *duplex Y arc* in the samples from the primase-deficient infection indicates that some of the concatemer tails from the rolling circles are completely duplex. Some lagging strand synthesis apparently occurs even in the absence of gp61, presumably through the action of another primase or by some alternative mechanism. Several models can be considered. First, the host DnaG primase might substitute for gp61. However, origin plasmid replication still occurs when a T4 *6I* deletion mutant infects a host *dnaG* temperature-sensitive mutant under non-permissive conditions, arguing against an involvement of the host primase (14,19). Second, T4 may encode another DNA primase, perhaps a late gene product, to explain the replication delay in primase-deficient infections. Third, Mosig *et al.* (19) proposed that endonuclease VII (gp49) cleaves recombinational junctions in T4 DNA in *6I* mutant infections and thereby provides primers for recombination-dependent replication. While this model could explain phage genomic replication, we cannot imagine how it could apply to origin plasmid replication. Fourth, RNA polymerase-generated transcripts that are able to hybridize to the single-stranded template may act as primers for lagging strand synthesis when gp61 is absent. We are currently defining the T4 gene products required for growth in the absence of gp61 by analyzing mutations that are synthetically lethal with the gene *6I* deletion. With these and other experiments, we hope to solve the mystery of DNA replication in the absence of the gp61 primase.

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