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Replication of Phage fd RF with fd Gene 2 Protein and Phage T4 Enzymes*

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Thomas F. Meyer‡§, Irmtraud Bäumel§, Klaus Geider§, and Patricia Bedinger¶

From the § Max-Planck Institut für medizinische Forschung, Abteilung Molekulare Biologie, Jahnstrasse 29, D6900 Heidelberg, Federal Republic of Germany, and the ¶ Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143

Bacteriophage fd replicative form DNA with a nick in the viral strand serves as a template for DNA replication with purified bacteriophage T4 enzymes. As anticipated from previous *in vitro* studies carried out with this system (Morris, C. F., Sinha, N. K., and Alberts, B. M. (1975) *Proc. Natl. Acad. Sci. U. S. A.* 72, 4800-4804), DNA is synthesized by a rolling circle mechanism. We show here that the DNA strands synthesized are processed by the phage fd gene 2 protein into unit length products, providing that the gene 2 protein is present at the moment when this DNA is made. The products are mostly unit length linear single strands, indicating that the circularization step normally catalyzed by gene 2 protein subsequent to its site-specific cleavage of an fd DNA strand occurs only inefficiently in this system. The gene 2 protein reduces the level of DNA synthesis by 2-fold at low concentrations, even though it only cleaves the DNA products efficiently at higher levels of the enzyme. This indicates that there are at least two different effects of the fd gene 2 protein in processing of viral fd DNA.

Phage fd gene 2 protein is required for replication of double-stranded fd DNA (1, 2). Its isolation as a biologically active enzyme (3) has permitted a detailed analysis of its biochemical properties (4). Striking features are its action as a site- and strand-specific endonuclease (5) and its ability to act in a reversible manner resealing a previously nicked fd RF[1] (4). In addition to creating the nick at which viral DNA replication begins, the gene 2 protein is responsible for generating unit length viral single-stranded DNA circles in place of the long linear concatemeric DNA which would otherwise be made during viral DNA replication (6). Since the required cleavage and circularization reactions can be accomplished with gene 2 protein alone when the viral strand is replicated *in vitro* by phage T7 replication enzymes, this processing of the replicating viral fd DNA appears not to require host enzymes.

A major obstacle during the previous studies carried out with the phage T7 *in vitro* DNA replication system was the tendency of the T7 DNA polymerase to switch strands, which diminished the yield of single-stranded DNA drastically (6). In this report, the T4 phage-encoded *in vitro* DNA replication system (7) has been used to catalyze fd single-stranded DNA replication in place of *Escherichia coli* DNA polymerase III holoenzyme, *E. coli rep* protein, and *E. coli* DNA-binding protein I (8).[2] This has allowed a more complete characterization of intermediate steps in the processing of fd viral strands by the gene 2 protein.

The phage T4 multienzyme *in vitro* system has been recently shown to replicate single-stranded phage ϕX174 DNA with high accuracy, with more than 30-fold net synthesis (9, 10). This system is also able to start DNA synthesis on a nicked double-stranded DNA template (7). Phage fd RF cleaved by gene 2 protein provides an appropriate substrate for this T4 DNA replication system since the fd enzyme does not remain attached to the cleaved DNA (4, 5), in contrast to the otherwise analogous phage ϕX RF cleaved with the ϕX174 gene A protein (11-13).

EXPERIMENTAL PROCEDURES

The isolation of bacteriophage fd gene 2 protein and phage fd RF I has been described (3). The phage T4 proteins were prepared as described elsewhere (7). *E. coli* DNA polymerase I was obtained from *E. coli* cells as described (3).

Replication of fd RF was performed in 100-μl volumes containing 17 mM Tris-acetate (pH 7.8), 33 mM K acetate, 5 mM Mg acetate, 10% glycerol, 0.1 mg/ml of bovine serum albumin, 0.5 mM dithiothreitol, 0.5 mM ATP, 0.2 mM concentration each of dATP, dCTP, and dGTP, and 0.04 mM [α-^{32}P]dTTP (4×10^6 cpm/assay). As template, 0.3 μg of fd RF was used. The phage T4 replication proteins added were 0.34 μg of T4 DNA polymerase, 7.3 μg of gene 32 protein, 2.8 μg of gene 44/62 protein, and 3.4 μg of gene 45 protein. Incubation was at 30 °C for 30 min.

Phage fd RF II_0 was prepared by treatment of fd RF I with 0.08 unit of gene 2 protein in the assay mixture described above in the absence of T4 proteins and the radioactive label. Incubation was for 15 min at 30 °C. The gene 2 protein was inactivated by heating to 50 °C for 5 min. Phage fd RF II was derived from fd RF II_0 by a nick-translation reaction (14) carried out with 1 μg of *E. coli* DNA polymerase I for 20 min at 20 °C. This results in a random position of the nick in the viral strand.

For electron microscopy, the samples were phenol extracted and the DNA was spread according to Davis *et al.* (15). Agarose gel electrophoresis was carried out as described previously (4).

RESULTS

Phage fd RF I DNA is converted to an efficient template for the T4 *in vitro* DNA replication system after it has been nicked by the fd gene 2 protein. When the gene 2 protein is inactivated prior to the start of DNA synthesis, this nicked template allows a level of DNA synthesis at least 7-fold above

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‡ Present address, Cold Spring Harbor Laboratory, P. O. Box 100, Cold Spring Harbor, New York 11724.

[1] The abbreviations used are: RF, replicative form; RF I, doubly closed, supercoiled replicative form DNA; RF II_0, RF specifically cleaved in the viral strand by gene 2 protein (5); RF II, RF with a random nick in the viral strand.

[2] T. F. Meyer and K. Geider, manuscript in preparation.

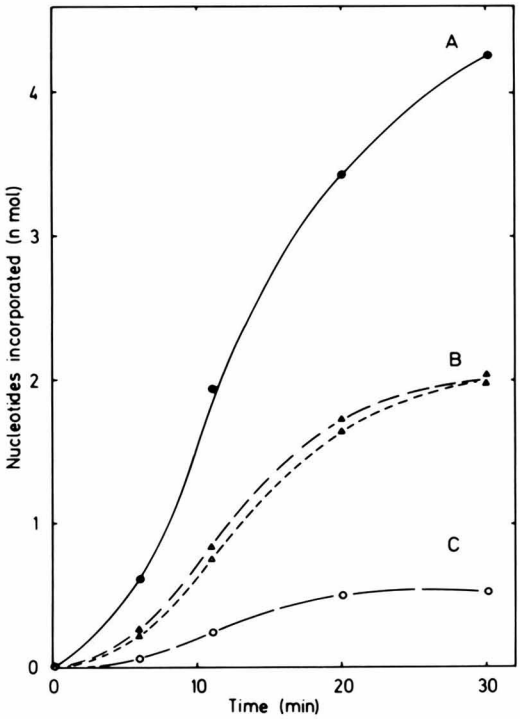

FIG. 1. **Kinetics of nucleotide incorporation into fd RF with phage T4 specific proteins.** *A*, fd RFII$_0$ without fd gene 2 protein; *B*, fd RFII$_0$ (▲) or fd RFII (△) with gene 2 protein (0.08 unit); C, fd RFI. Aliquots were removed at the time indicated and acid-insoluble radioactivity was measured.

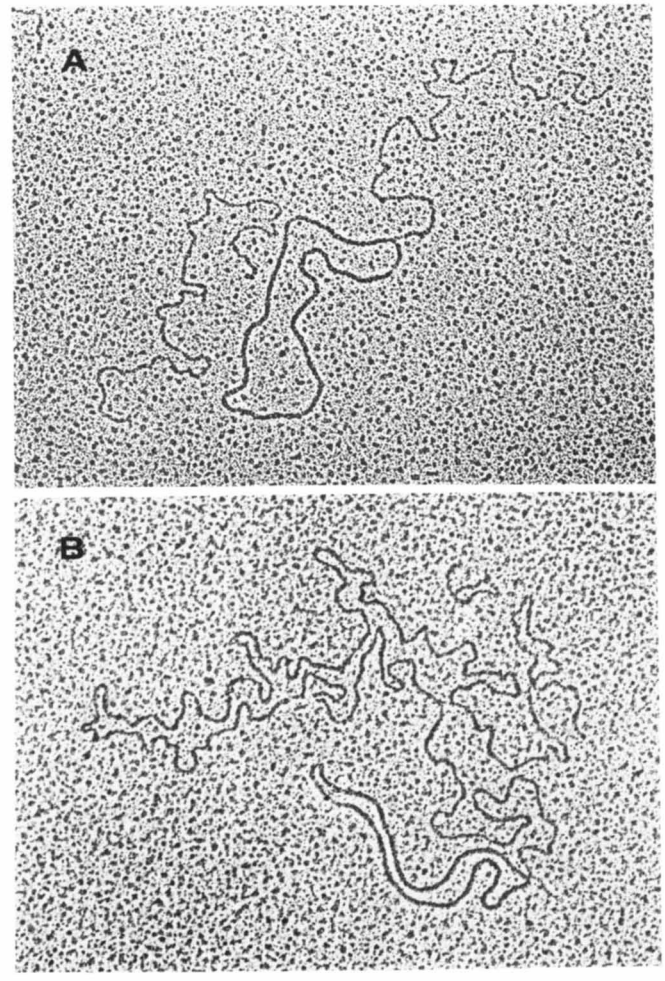

FIG. 2. **Electron microscopic pictures of typical replication products.** *A*, a rolling circle with a single-stranded tail of about unit length besides a linear molecule of unit length. *B*, a rolling circle with a tail of multiple genome length. The incubation conditions for *A* and *B* are as described in Fig. 3, *B* and *E*, respectively. Pictures were taken with the electron microscope Philips EM 400 at the magnification of: × 8350.

that obtained using an untreated fd RF I preparation (Fig. 1, *curves A* and *C*) comprising 8-fold net synthesis of viral DNA; incorporation with the untreated DNA arises from about 10% randomly nicked molecules. Analysis of these reaction products by electron microscopy shows that replication occurs in a rolling circle mode, beginning by covalent addition of nucleotides onto the 3′OH end of the nick, with concomitant parental strand displacement as the polymerase moves in the 5′ → 3′ direction. Without active gene 2 protein present during the DNA synthesis, the rolling circle products have single-stranded tails of greater than unit length (Fig. 2*B* and Table IC). These long single-stranded tails are resistant to processing by gene 2 protein when the enzyme is added postreplicatively (Fig. 3, *D* and *E*).

If active gene 2 protein is included in replication reactions, the level of *in vitro* DNA synthesis is inhibited 2-fold compared to the reaction without active gene 2 protein present (Fig. 1, *curve B*). This inhibition occurs regardless of whether initiation takes place at the cleavage site of gene 2 protein (RF II$_0$) or at a random nick in the viral strand of fd RF (RF II) (Fig. 1, *curve B*). Recent observations[3] indicate that gene 2 protein binds only to the specific nick at the origin of viral strand replication, but not to otherwise located single strand breaks. Therefore, the observed inhibition by gene 2 protein is apparently not due to direct interference at the site of initiation of DNA synthesis with the T4 enzymes. A clue to this 2-fold inhibition by active gene 2 protein has been provided by electron microscopy (Table IA and Fig. 2*A*). In a reaction including gene 2 protein, rolling circle structures with a single-stranded tail of unit length are observed frequently compared to rolling circle structures shorter than unit length. This suggests that the replication fork pauses at the gene 2 protein recognition site before the strand cleavage reaction. This pause is believed to account for the 2-fold slower average rate replication in the presence of active gene 2 protein (see "Discussion").

Analysis of the replication products made in the presence of active gene 2 protein, using agarose gel electrophoresis and autoradiography, shows incorporation of radioactive precursor into DNA species migrating in the positions expected for nicked double-stranded circles and unit length single-stranded linear molecules and, to a lesser extent, the production of unit length single-stranded circular molecules (Fig. 3, *A–C*). Electron microscopy confirms these findings by showing the accumulation of unit length single-stranded linear molecules as the major product (Table I, A and B), rather than longer single-stranded tails attached to circles. This shows that when active gene 2 protein is present in these reactions, efficient cleavage at the viral strand origin takes place. Analysis by gel electrophoresis (Fig. 3, *F* and *G*) and electron microscopy (Table IB) reveals almost identical reaction products whether the initial template contains the specific gene 2 protein nick or a random nick, indicating that a specific 5′ end is not required for the gene 2 protein cleavage reaction.

The efficiency with which the gene 2 protein catalyzes circularization of the unit length single strands produced is very poor in the T4 replication system since only about 10% of the product single strands are circles (Fig. 4). In contrast, in similar *in vitro* experiments carried out in the *E. coli* DNA

[3] K. Geider, I. Bäumel, and T. F. Meyer, manuscript in preparation.

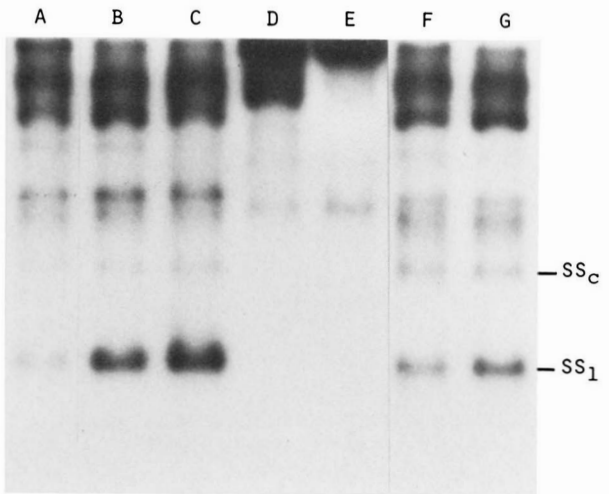

FIG. 3. **Agarose gel of the replication products.** The positions of unit length linear fd DNA (SS_l) and single-stranded circles (SS_c) are indicated. Replication was as described under "Experimental Procedures." RFI was used as template in the experiments *A–C*. Gene 2 protein was present for 0.08 unit (*A*), 0.3 unit (*B*), and 0.6 unit (*C*). Experiments *D* and *E* were with fd $RFII_0$ without gene 2 protein. Experiments *F* and *G* were with fd RFII and 0.08 unit of gene 2 protein (*F*) or 0.3 unit of gene 2 protein (*G*). After incubation for 30 min at 30 °C, the samples were prepared for gel electrophoresis, except for sample E which was further heated for 5 min at 65 °C to inactivate T4 replication enzymes and then treated with 0.3 unit of gene 2 protein for 15 min at 30 °C before application to electrophoresis.

TABLE I

Replication products seen in the electron microscope

Replication was as described under "Experimental Procedures" without or with gene 2 protein (0.6 unit). A, $RFII_0$ with gene 2 protein; B, RFII with gene 2 protein; C, $RFII_0$ without gene 2 protein.

Type	A	B	C
Rolling circles of about unit length	12%	11%	2%
Of less than unit length	12%	12%	3%
Of larger than unit length	3%	5%	43%
Replicative form DNA	31%	32%	14%
Linear single strands of unit length	28%	26%	4%
Other single-stranded molecules	14%	14%	34%
No. of molecules counted	241	150	228

replication system[2] and in the phage T7 (6), the major products were unit length single-stranded circles. This suggests that one or more of the protein components in the T4 replication system may be inhibiting the circularization reaction.

Increasing the gene 2 protein concentration increases the level of cleavage products (Fig. 4, *curve B*). However, even at very low concentrations, where the cleavage reaction is inefficient, the gene 2 protein inhibits DNA synthesis 2-fold, a level which does not increase even at high gene 2 protein concentrations (Fig. 4, *curve A*). These data suggest that a single gene 2 protein molecule binds to the DNA (at the origin of viral strand replication) causing inhibition of DNA synthesis in the T4 *in vitro* system, while at least 2 molecules of gene 2 protein must bind to the origin sequence to achieve cleavage (see below).

DISCUSSION

The data in this paper demonstrate that fd RF I molecules nicked by fd gene 2 protein are utilized by T4 replication proteins in *in vitro* DNA synthesis reactions. When active gene 2 protein is not included in replication reactions, the products are rolling circles with long single-stranded tails. Although these long tails contain recognition sequences for gene 2 protein, they are not substrates for gene 2 protein

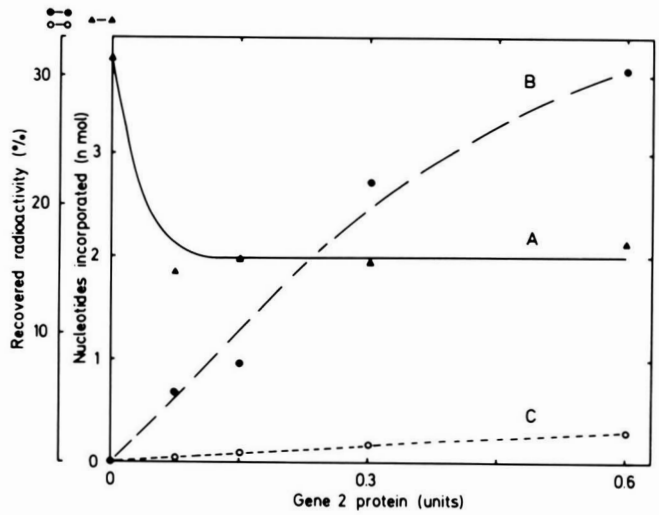

FIG. 4. **The effect of gene 2 protein on the synthesis of single-stranded linear and circular DNA and its influence on DNA replication.** The standard mixture was supplemented with fd $RFII_0$ and increasing amounts of gene 2 protein. DNA synthesis (*A*) was measured by determination of acid-insoluble radioactivity. Linear single strands (*B*) and circular single strands (*C*) were determined after separation of the replication products on an agarose gel and subsequent counting of gel slices.

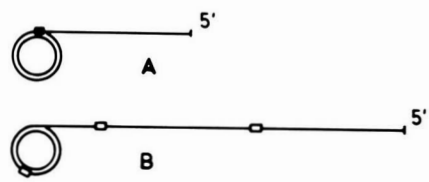

FIG. 5. **A model for cleavage sites of fd gene 2 protein on rolling circles of fd RF.** The rolling circle intermediate A bears an active cleavage site, as the replication fork passed the gene 2 protein recognition site. Recognition sequences in relaxed double-stranded DNA and in single-stranded DNA are inactive for cleavage (4), as pointed out in intermediate B. ■, Active site; □, inactive site.

cleavage, just as single-stranded fd molecules are inert as cleavage substrates (4). Apparently cleavage at gene 2 protein sites requires that the site be in a topologically altered conformation. This can be created by negative supercoiling of double-stranded circular DNA molecules (4). We propose that a similar DNA conformation is created just ahead of a replication fork as the helix begins to unwind to allow leading strand DNA synthesis, thereby activating cleavage by the gene 2 protein (Fig. 5).

The predominant products of replication reactions which include active gene 2 protein are single-stranded unit length linear molecules. Mostly circular unit length products are formed in *E. coli*[2] and T7 (6) *in vitro* DNA replication systems. Thus, the circularization reaction which normally follows cleavage by the gene 2 protein is drastically reduced in the T4 *in vitro* replication system. While it is possible that this is due to the lack of some critical circularization factor which is present in the other two *in vitro* systems, it seems more likely that competition by the T4 proteins for the two DNA ends to be joined prevents the gene 2 protein from circularizating the cleaved molecules in this system. The presence of T4 gene 32 protein (T4 DNA-binding protein) might be unfavorable for the circularization reaction.

Gene 2 protein apparently functions in at least two modes in the T4 *in vitro* replication system. One causes inhibition of DNA replication by interfering with strand unwinding or with polymerase movement through the double helical template at the gene 2 protein recognition site; another leads to specific

DNA strand cleavage. A 2-fold inhibition of DNA synthesis is caused by gene 2 protein at a low gene 2 protein concentration, while the efficiency of cleavage increases with increasing gene 2 protein concentrations. The electron microscopy suggests that inhibition is caused by a pausing of the replication fork at the gene 2 protein-bound origin site (Table I). Cessation of DNA replication near the origin of replication was also anticipated recently *in vivo* for phage f1 (16).

The palindrome structure of the gene 2 protein cleavage site (5) may attract 2 gene 2 protein molecules in case of fd RF I or for a rolling circle intermediate resting after an almost full round of replication. Such a complex is considered active for strand cleavage. After the cleavage reaction, 1 gene 2 protein molecule remains bound to the complementary strand, according to the finding that this strand is protected against nuclease treatment at the site where cleavage occurs.[3] The binding of this gene 2 protein molecule to the complementary strand might be responsible for the cessation of DNA replication after each round and might prepare the rolling circle intermediate for cleavage by a second molecule of gene 2 protein.

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