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Origin of DNA replication of bacteriophage f1 as the signal for termination

(duplication of origin/plus-strand synthesis/rolling circle/deletion)

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ABSTRACT **Restriction fragments that contain the origin of DNA replication of bacteriophage f1 were inserted *in vitro* into circular f1 DNA molecules to form genomes that contain two origins. This DNA was used to transfect *Escherichia coli*. Analyses of the DNA of the progeny phage indicated that one origin and the DNA segment located between the two origins in the infecting DNA molecules had been eliminated. This result is interpreted to mean that the nucleotide sequence of the origin for plus (viral)-strand synthesis also serves as the signal for the termination of DNA synthesis.**

DNA replication of single-stranded DNA phages, either icosahedral or filamentous, occurs in three steps in infected cells (for review, see refs. 1 and 2): (*i*) conversion of the parental viral DNA into double-stranded replicative form (SS→RF), (*ii*) replication of the replicative form DNA (RF) to form a pool of progeny RF molecules (RF→RF), and (*iii*) asymmetric replication of single-stranded viral DNA on the RF template (RF→SS). A model has been proposed in which only two mechanisms are required for all three steps to proceed (3–5): (*i*) the synthesis of a minus (complementary) strand on a single-stranded plus (viral)-strand template, which results in the formation of a double-stranded RF molecule, and (*ii*) the synthesis of a plus strand on a double-stranded template, in which the newly synthesized plus strand displaces the old plus strand on the RF, resulting in the formation of a single-stranded old plus strand and a double-stranded RF molecule.

For filamentous phages such as f1, M13, or fd, the origin of minus-strand synthesis (minus-origin) and that of plus-strand synthesis (plus-origin) are located close to each other on the genome, in the middle of the large intergenic space between genes IV and II (3) (see Fig. 1). Replication of both the plus and the minus strands proceeds in a 5′ → 3′ overall direction (3). After one round of synthesis of either minus or plus strand, the double-stranded molecule formed is converted to a closed circle (RF IV) and then to a superhelical form (RF I) before the next round of synthesis starts (12).

Replication of the minus strand is initiated by the synthesis of some 30 bases of primer RNA by host RNA polymerase at the minus-origin (8). Upon completion of one round of minus-strand synthesis, the elongating 3′ end will reach the 5′ end of the same strand, and the strand will be closed. Thus, the signal for the termination (i.e., closure) of minus strand may well be the presence of its own 5′ end, not a particular nucleotide sequence.

The replication of the plus strand, on the other hand, is initiated by introduction of a nick (single-strand break) at the specific site (plus-origin) on the plus strand of the RF I molecule by the action of gene II protein (or cisA protein in the icosahedral phages) (9, 13). Synthesis proceeds by elongation of the 3′ end thus produced, and the old plus strand is displaced in a single-stranded form starting from its 5′ end [rolling circle model (14)]. How does the replication complex know where to terminate synthesis and close the plus strand? In the *in vitro* replication of the plus strand of φX174, the 5′ end is bound to cisA protein (13, 15, 16) and appears to travel with the replication fork along the RF molecule (16). Thus, it is conceivable that the nucleotide sequence at the plus-origin may be recognized by the gene II (or cisA) protein at the end of one round of replication as the signal for the termination of replication.

To test this hypothesis, I constructed phage DNAs *in vitro* that had a duplication of the origins. Upon transfection with such DNA molecules, the gene II protein should initiate phage DNA replication at either one of the two plus-origins on the genome. If the nucleotide sequence of the plus-origin also serves as the signal for the termination, plus-strand synthesis will terminate when the replication fork reaches the other plus-origin; a circular viral strand shorter than the parental genome will be produced and will serve as the template for further replication. The region of the genome deleted will be precisely that which was located between the two plus-origins. On the other hand, if termination occurs at the site where replication initiated, the progeny phage will contain the duplication. The results described in the present paper indicate that the nucleotide sequence at the plus-origin also serves as the signal for the termination of DNA synthesis.

MATERIALS AND METHODS

R199 and R218 (a clear plaque-forming derivative of R209) are two insertion mutants of phage f1 and harbor a single *Eco*RI site at the *Hae* III F/G border and at the G/D border, respectively, as described by Boeke *et al.* (17) (see Fig. 1). R220 (J. D. Boeke, G. F. Vovis, and K. Horiuchi, unpublished data) is a mutant of f1 which contains two *Eco*RI sites. The R220 *Eco*RI B fragment is the smaller of the two DNA fragments produced by treatment of R220 RF with *Eco*RI and includes the region between *Hpa* II A/H border and *Hae* III G/D border (see Fig. 1). *Escherichia coli* strains K38 (18) and K507 (17) have been described.

Restriction enzymes *Hae* III (19) and *Hin*fI (20) were prepared as described. *Eco*RI was purchased from New England BioLabs. T4 DNA ligase and alkaline phosphatase were purchased from Bethesda Research Laboratories (Rockville, MD) and Worthington, respectively. Conditions for the enzyme reactions have been described (17, 19, 20). Transfection of $CaCl_2$-treated *E. coli* cells (21) and gel electrophoretic analysis of restriction digests were carried out as described (19). RF molecules were prepared by a simplified method (22).

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Abbreviations: RF, replicative form DNA; DI particles, defective interfering particles.

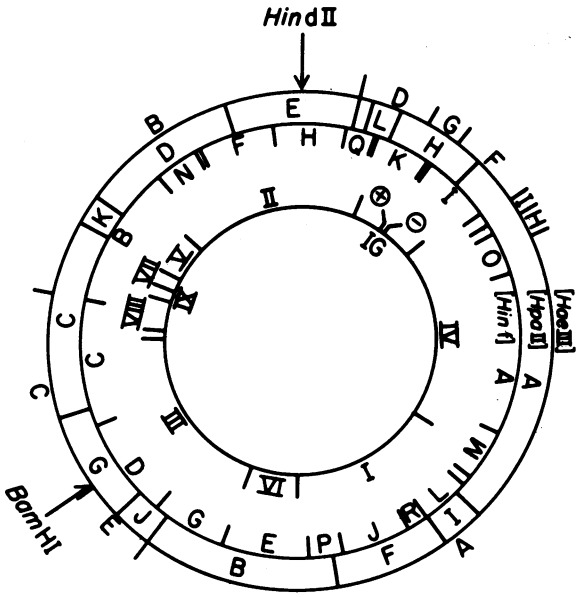

FIG. 1. Restriction map of bacteriophage f1. The outer circles, representing the physical map, show the cleavage sites of restriction enzymes *Hae* III, *Hpa* II, and *Hin*fI. The single *Hin*dII and *Bam*HI cleavage sites are indicated by arrows. The inner circle represents the genetic map. Genes are indicated by roman numerals. IG refers to the intergenic space between genes II and IV (6, 7). ⊕ and ⊖ represent the origins of replication of plus and minis strands, respectively (3, 8, 9). The 5′ → 3′ direction of the plus strand is counterclockwise on this map. For construction of the map, see refs. 10 and 11.

RESULTS

Construction of DNA Molecules with Duplicated Origin. The origin of DNA replication of phage f1 is located within the restriction fragment *Hae* III G (3) (see Fig. 1). The f1 mutants R199 and R218 harbor a single *Eco*RI site at the *Hae* III F/G border and at the G/D border, respectively (17). RF I molecules of R199 and R218 were cleaved with *Eco*RI, and the full-length linear duplex (RF III) molecules produced were treated with alkaline phosphatase. Short restriction fragments containing the f1 origin and flanked by *Eco*RI sticky ends were added to the RF III preparations; the mixtures were ligated with T4 DNA ligase and then used for transfection. A fragment used (R220 *Eco*RI B fragment) included the region between *Hpa* II A/H border and *Hae* III G/D border (Fig. 1)—i.e., *Hae* III G plus a portion of *Hae* III F (called F′ in this paper)—and was flanked by *Eco*RI sticky ends (see Fig. 2).

When R220 *Eco*RI B fragment was mixed with the *Eco*RI- and phosphatase-treated R199 or R218 RF and ligated, infectious DNA molecules were produced. Transfection data shown in Table 1 indicate that addition of the R220 *Eco*RI B fragment stimulated the production of infectious molecules from the phosphatase-treated RF III by 6- to 30-fold. Analysis of the ligation products on a 0.8% agarose gel indicated that the phosphatase-treated RF III remained as RF III when treated with ligase by itself (23) but were converted to multimers and circles when treated with ligase in the presence of the R220 *Eco*RI B fragment (data not shown).

Characterization of Progeny Genomes. From Exp. 1 (R199 RF III was used) and Exp. 3 (R218 RF III was used) shown in Table 1, eight single plaques (1-1, 1-2, . . . , 1-8; 3-1, 3-2, . . . , 3-8) were picked and purified. Their relative plating efficiencies on bacterial strains K507 ($r_{RI}^+m_{RI}^+$) compared to K38 ($r_{RI}^-m_{RI}^-$) showed that all the isolates tested were restricted to the same extent as their parent, which was used in the form of RF III (0.2 for R199, 0.4 for R218; data not shown). Because the plating efficiency of f1 phage with two *Eco*RI sites is not more than 0.05 (J. D. Boeke, G. F. Vovis, and K. Horiuchi, unpublished data), this result indicated that the progeny phages had only the one *Eco*RI site characteristic of their parent. This is to be expected were replication to be terminated at the origin sequence (see Fig. 2).

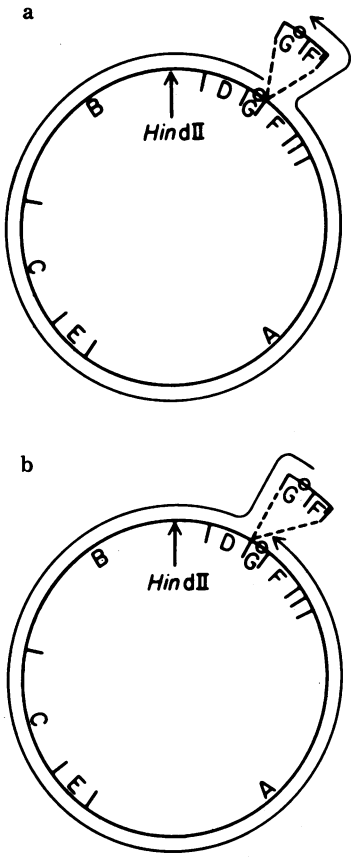

FIG. 2. Diagrams showing the design of an experiment to test whether the origin of DNA replication signals termination. The large circles represent the genomes of f1 mutants R199 (*a*) and R218 (*b*), which are divided into *Hae* III restriction fragments. The small circles on the *Hae* III G fragment represent the origin of replication of the plus strand. The R220 *Eco*RI B restriction fragment consists of *Hae* III G fragment and F′ fragment, a portion adjacent to *Hae* III G of *Hae* III F fragment. The R220 *Eco*RI B fragment is flanked by the *Eco*RI sticky ends. In *a*, this fragment was inserted into the single *Eco*RI site of R199—i.e., at the border between *Hae* III F and G. In *b*, it was inserted into the single *Eco*RI site of R218—i.e., at the border between *Hae* III G and D. Self-recircularization of linear DNA molecules of R199 and R218 was prevented by treatment with alkaline phosphatase (23). The constructed DNA molecules were used to transfect *E. coli* strain K38, and plaques obtained were picked and purified. Their RF were analyzed by cleavage with restriction enzymes. If the nucleotide sequence of the origin of plus-strand synthesis is also to serve as the signal for termination of the synthesis (i.e., closure), the genome of the progeny phage should be shorter than the parental genome, as indicated by long curved arrows. In *a*, the progeny genome should contain a tandem duplication of segment F′ with a single *Eco*RI site between F and F′; in *b*, the progeny should be the size of the unit-length genome with a single *Eco*RI site between G and D.

As diagrammed in Fig. 2, if the loss of an *Eco*RI site is due to the premature termination of DNA replication at the origin, the progeny phages that were derived from R199 RF III and the R220 *Eco*RI B fragment should have a tandem duplication of the F′ segment (Fig. 2*a*) and the progeny phages derived from R218 RF III and the R220 *Eco*RI B fragment should be identical to R218 (Fig. 2*b*). Restriction enzyme analyses of RF I of isolates 1-1 through 1-8 and 3-1 through 3-8 showed precisely this pattern. Fig. 3 shows the gel pattern of the *Hin*fI digests. The phage progeny derived from R199 RF III and the

Table 1. Infectivity of *Eco*RI-digested DNA samples after ligation

DNA*	Amount, fmol†	No. of plaques	Infectivity of DNA, %‡
1. Lig(R199·*Eco*RI·AP + R220 *Eco*RI B)	65	≈1000	1.0
2. Lig(R199·*Eco*RI·AP)	65	35	0.03
3. Lig(R218·*Eco*RI·AP + R220 *Eco*RI B)	50	310	0.4
4. Lig(R218·*Eco*RI·AP)	50	54	0.07
5. Lig(R220 *Eco*RI B)	200	0	0
6. Wild-type f1 RF I	0.1	152	100
7. None	0	0	—

$CaCl_2$-treated *E. coli* Hfr strain K38 was transfected with the DNA samples as indicated and plated for infective centers.

* Lig, DNAs shown in parentheses were incubated with T4 DNA ligase; ·*Eco*RI·AP, treated with *Eco*RI and then with alkaline phosphatase.

† Amount of RF III molecules per plate is indicated. In Exps. 1 and 3, 200 fmol of the R220 *Eco*RI B fragment was present in addition.

‡ Number of plaques was divided by the amount of DNA and then normalized to the value obtained with f1 RF I.

R220 *Eco*RI B fragment (lanes 1–5) had a novel pattern. A unique fragment (designated I′) has replaced the *Hin*fI I fragment. The size difference between fragments I′ and I was about 115 base pairs, in agreement with the size of F′ (the region between the *Hpa* II A/H border and the *Hae* III F/G border). However, the phage progeny derived from R218 RF III and the R220 *Eco*RI B fragment (lanes 6–10) showed a pattern identical to that of wild-type f1 (lane 11) (R199 and R218 gave a pattern identical to f1). Thus, the phage progeny derived from R199 RF III have a tandem duplication of F′ at the *Hae* III F/G border, and those derived from R218 RF III are identical to R218 (see Figs. 1 and 2).

The gel electrophoretic patterns of *Hae* III digests and *Hae* III plus *Eco*RI digests shown in Fig. 4 confirm this conclusion. RF preparations from the phages produced by ligation of R199 RF III and the R220 *Eco*RI B fragment yielded, upon treatment

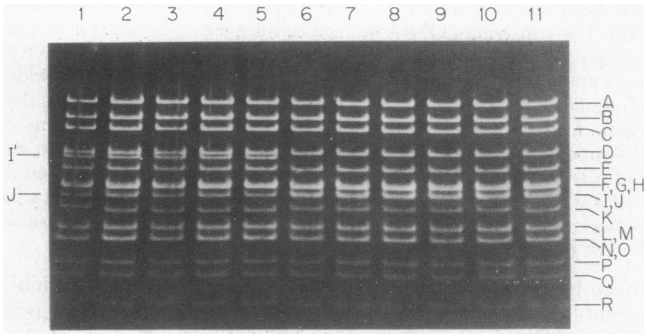

FIG 3. *Hin*fI restriction cleavage of the progeny phage DNA. Substrate RF preparations used were of the following phages. Lanes 1–5, five isolates of progeny phages (1-1 through 1-5, respectively) obtained by transfection with the DNA sample in which the R220 *Eco*RI B fragment was ligated with the R199 RF linearized with *Eco*RI (see Fig. 2*a*). Lanes 6–10, five isolates of progeny phages (3-1 through 3-5, respectively) obtained in a parallel experiment in which R218 was used instead of R199 (see Fig. 2*b*). Lane 11, wild-type f1. The RF of each phage was prepared as described (22), digested with *Hin*fI, and electrophoresed on a 2.5–7.5% acrylamide gradient gel (19). The gel was stained with 1 μg of ethidium bromide per ml and photographed under UV light. The *Hin*fI restriction fragments of wild-type f1 (see Fig. 1) are indicated on the right of the figure. A novel fragment I′, which is about 115 bases longer than I, is indicated on the left of the figure.

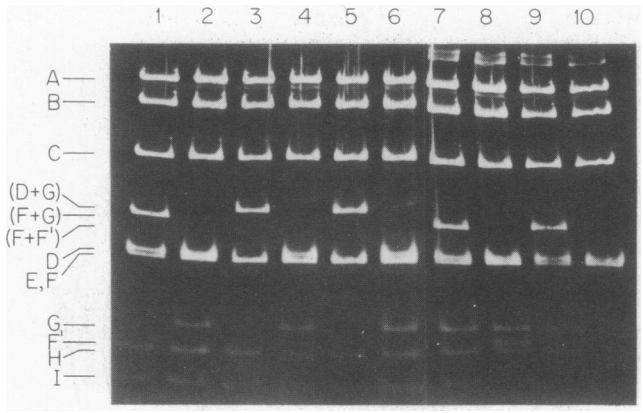

FIG. 4. Restriction cleavage analysis with *Hae* III and *Eco*RI of the progeny phage genome. The substrate RF preparations used were of the following phages. Lanes 1 and 2, R199; lanes 3 and 4, R218; lanes 5 and 6, an isolate of progeny phages (3-1) obtained by transfection with the DNA sample in which the R220 *Eco*RI B fragment was ligated with R218 RF linearized with *Eco*RI (see Fig. 2*b*); lanes 7 and 8 and 9 and 10, two isolates of progeny phages (1-1 and 1-2, respectively) obtained by transfection with the DNA sample in which the R220 *Eco*RI B fragment was ligated with R199 RF linearized with *Eco*RI (see Fig. 2*a*). The samples in lanes 1, 3, 5, 7, and 9 were digested with *Hae* III alone; the samples in lanes 2, 4, 6, 8, and 10 were digested with *Hae* III plus *Eco*RI. Procedures for RF preparation, gel electrophoresis, and visualization of the bands were the same as in Fig. 3. The *Hae* III restriction fragments of f1 are indicated on the left of the figure. F′ represents a segment of the f1 genome located between the *Hpa* II A/H border and the *Hae* III F/G border.

with *Hae* III, a free *Hae* III G fragment and a unique fragment (F + F′). The latter was converted into *Hae* III F and a novel fragment F′ upon further digestion with *Eco*RI. This result strengthens the conclusion that the phage genome contains a tandem duplication of the F′ segment with the single *Eco*RI site located between F and F′ (see Fig. 2*a*). RF preparations from the phages produced from R218 RF III and the R220 *Eco*RI B fragment gave exactly the same restriction pattern as R218. Digestion with *Hae* III did not produce *Hae* III D and G fragments, but produced a unique fragment (D + G), which was converted into fragments *Hae* III D and G by further digestion with *Eco*RI. Thus, the single *Eco*RI site is located at the *Hae* III G/D border (see Fig. 2*b*).

DISCUSSION

The results described above strongly support the notion that the nucleotide sequence of the origin of plus-strand synthesis also serves as the signal for termination of plus-strand synthesis. This property necessarily prohibits the presence of two plus-origins in one genome. When a genome that contains two plus-origins is constructed *in vitro*, it always loses one origin together with the segment between the two origins upon DNA replication. Several different restriction fragments of f1 other than the R220 *Eco*RI B fragment have been inserted *in vitro* into the *Eco*RI site of R199 or R218. Whenever the fragment used contained the origin of DNA replication (e.g., the *Hae* III G fragment flanked by *Eco*RI sticky ends), the progeny phages were found to have lost one origin and the segment of DNA located between the two origins (data not shown).

The experimental data presented above do not allow us to distinguish critically the role of the plus-origin and the minus-origin. However, as discussed previously, it is the origin of the plus strand, from which replication occurs by the rolling circle mechanism, that should serve as the termination signal. The termination signal of the minus strand may well be its own 5′ end. Moreover, experimental support is available from the

work of Kaguni and Ray (24), who introduced the minus-origin of phage G4 into the M13 genome and showed that this phage DNA can replicate in the presence of rifampicin by using the G4 origin for initiation of minus-strand synthesis. This means that the M13 minus-origin does not signal termination of replication. Thus, it must be the plus-origin that is not allowed to exist in duplicate on a genome.

Defective interfering (DI) particles of filamentous phages (25–27) often harbor a tandem reiteration of regions near the origin of DNA replication (7). Nucleotide sequence analysis of these DI particles showed that the reiterated segments contained the minus-origin and ended at or very near the plus-origin (28). For some of the DI particles it was shown that the reiterated segments did not contain a functional plus-origin (29). The present results predict that none of the reiteration would involve a functional plus-origin. The advantage in DNA replication of the DI particles with the reiteration (7) might have to be attributed to the existence of multiple minus-origins.

In the present experiment, if the R220 *Eco*RI B fragment were inserted into the RF III molecules in a direction that was opposite to its natural orientation, there should be no premature termination of DNA replication. However, progeny phages having such a structure have not been found. It would appear that a genome having such an inverted repeat is not viable.

We saw that the insertion of fragment G-F′ into the G/F border resulted in the production of phages with a duplication of F′. Simultaneously, the segment between the two origins was eliminated from the infecting DNA molecules. By using this principle one should be able to construct various mutant phage genomes containing either duplications or deletions near the origin of DNA replication. Such mutants might be useful in the analysis of the nucleotide sequence elements necessary for the function of the origin. It can be predicted that if foreign DNA containing the nucleotide sequence of the plus-origin were inserted, for gene cloning, into the phage genome, it would result in a deletion.

Some DNA genomes with multiple origins are known to exist: there are DI particles of simian virus 40 that contain multiple reiteration of the origin of DNA replication (30), and multimeric forms of plasmid DNA are often observed in *E. coli* cells. Therefore, the recognition of the origin of DNA replication as termination signal may be characteristic of instances where DNA replicates by a rolling circle mechanism.

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