Note

Essential Structures in the Complementary DNA Origin of Bacteriophage G4

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G4 is a bacteriophage closely related to ϕ X174¹⁾ with single-stranded circular DNA of 5577 bases. The priming system for the initiation of synthesizing the complementary DNA strand of G4 is distinct from that of ϕ X174.^{2,3)} With phage ϕ X174, the priming system is rather complex and contains eight proteins. With phage G4, the single-

strand-binding (*ssb*) protein and the *dnaG* protein (primase) of *E. coli* are essential to synthesize the primer RNA molecules at the complementary origin of G4 (*Gori_c*) which contains three stems and loops I, II, and III (Fig. 1). The 274 base-pair *AluI* fragment from G4 RF DNA, which contains the entire *Gori_c* region in the intergenic space between the F and G genes,⁴⁾ was cloned in a correct or functional orientation to a filamentous phage vector, R199.⁵⁾ The chimeric phage R199/G4 carries the *Gori_c* region inserted at the *EcoRI* site in such a way that *EcoRI* sites were restored at both the ends of the *Gori_c* segment. The genomic DNA of R199/G4 carries a unique *AvaII* site at the foot of stem III in the *Gori_c* region.

A 10 base-pair *BamHI* linker (CCGGATCCGG) was inserted at the filled-in *AvaII*-cleaved site of R199/G4 RF DNA followed by transfection to *E. coli* JM103.⁶⁾ The resulting insertion mutant was named R199/G4-Bam4122 (Fig. 1). Another insertion mutant, R199/G4-Eco16, was constructed by inserting an 8 base-pair *EcoRI* linker (GGAATTCC) in the same manner. Actual DNA sequences in the altered region of each insertion mutant were confirmed by the Maxam-Gilbert sequencing method (Fig. 2(a), (b), and (c)). With the mutant Bam4122, the spacing between the stems II and III is 13 bases longer than that of the wild type *Gori_c* (Fig. 1). The mutant Eco16 carries two structural alterations (Fig. 1). First, the spacing between stems II and III is 10 base-pair longer than that of the wild type *Gori_c*. The G residue adjacent to the

FIG. 1. Schematic Representation of the Potential Secondary Structures of *Gori_c* wild type, Bam4122, and Eco16.

Numerals 3890, 3930 and 4010 indicate the nucleotide numbers on the G4 genome.¹⁰⁾ Base sequences involving a part of the stems and loops II and III are shown for Bam4122 and Eco16.

FIG. 2. DNA Sequences of Altered Tracts in the *Gori_c* Region of R199/G4 wild type, Bam4122, and Eco16. Base sequences of the viral strand (=V-strand) are given to the right of those of the complementary strand determined on the gel. Numerals 3930 and 3945 indicate the nucleotide numbers on the G4 genome.¹⁰⁾ (a) *Gori_c* wild type. (b) *Gori_c*-Bam4122. (c) *Gori_c*-Eco16.

FIG. 3. Formation of the Parental RF DNA in the Presence and Absence of Rifampicin.

Sedimentation is from right to left. Arrows with SS and RF indicate the location of single-stranded circular and replicative form DNAs as internal markers, respectively. In experiments (a), (b), (c), and (d), *E. coli* JM103 is infected with ³²P-labeled phages R199, R199/G4, R199/G4-Bam4122, and R199/G4-Eco16 in the absence of rifampicin, respectively. In experiments (e), (f), (g), and (h), the bacterium is infected with ³²P-labeled phages R199, R199/G4-Bam 4122 and R199/G4-Eco16 in the presence of 200 µg/ml rifampicin, respectively.

right-hand end of the *EcoRI* linker is missing for unknown reasons. Second, the presence of a G residue at the left end of the inserted *EcoRI* linker disrupts the lowermost GC base pair in stem III. Because of the unpaired A at 3936 and of the two mismatched Gs at 3894 and at 3938, the

mutant Eco16 possibly contains a stem III two base pairs shorter than that of wild type *Gori_c*. In other words the mutant Eco16 may contain aberrant structures at the foot of stem III.

Phages of R199, R199/G4, R199/G4-Bam4122, and

R199/G4-Eco16 were labeled with ^{32}P -phosphoric acid. The *Gori_c* activity was estimated by measuring the extent of parental RF formation upon infection by the phages of *E. coli* JM103 in the presence of 200 $\mu\text{g}/\text{ml}$ rifampicin. Experimental procedures for phage preparation and infection have been described.^{7,8)} The phage-bacterium complexes were killed by adding an equal volume of 2X poison buffer (20 mM NaN_3 /20 mM KCN/2 mM EDTA/20 mM Tris-HCl (pH 8.0)), washed three times with 1X poison buffer (10 mM NaN_3 /10 mM KCN/1 mM EDTA/10 mM Tris-HCl (pH 8.0)) and lysed with 6 mg/ml lysozyme and 1% Sarkosyl. The crude lysates were layered over 5 to 20% sucrose gradients and spun at 25,000 rpm for 15 hr at 4°C in an RPS-40T rotor of a Hitachi preparative ultracentrifuge 65P. After dropwise fractionation, the radioactivity in each fraction was measured in a liquid scintillation counter.

In the absence of rifampicin, the *ori_c* of R199 itself and possibly the *Gori_c* were functional (Fig. 3(a) and (b)). While the *ori_c* activity of R199 was inhibited in the presence of rifampicin, the *Gori_c* carried by R199/G4 was active even in the presence of the drug (Fig. 3(e) and (f)). Thus, the *Gori_c* function can be detected as a rifampicin-insensitive single-strand initiation (*ssi*) activity. The insertion mutant R199/G4-Bam4122 had diminished, but not completely abolished *Gori_c* activity (Fig. 3(c) and (g)). The insertion mutant R199/G4-Eco16 had almost no detectable *Gori_c* activity (Fig. 3(d) and (h)).

According to these experimental results, it is conceivable that the GC-rich tract centered in the *Ava*II site in the *Gori_c* cartridge is at least a part of the *Gori_c* determinant sequences. It is assumed that the potential secondary structures shown in Fig. 1 play essential roles in the *Gori_c*-directed priming events.⁴⁾ It is conceivable that the 7 base space between stems II and III is essential for the normal expression of *Gori_c* function at the wild type level. It is also conceivable that in addition to the presence of the proper

spacing between the stems II and III, the presence of the specific structure formed by the two GC base pairs at the bottom of stem III is essential to the functional *Gori_c* activity. This idea is quite consistent with the *in vitro* experimental results obtained with phage ϕK , which is closely related to phage G4.⁹⁾ Phage ϕK requires the same priming proteins as G4 for its *ori_c* to be functional. Sims and Benz demonstrated that the *E. coli* primase molecule binds to domains in the ϕK *ori_c* tract involving the bottom regions of and the spacing regions between stem structures in the functional *ori_c* priming complex.⁹⁾

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