

Structure of Inserted Bacteriophage Mu-1 DNA and Physical Mapping of Bacterial Genes by Mu-1 DNA Insertion

(electron microscopy/episomes/*Flac*/*E. coli*)

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ABSTRACT It is shown, by electron microscope observation of the structures of heteroduplexes, that Mu-1 DNA inserted into the bacterial episomes *Flac* and $F_8[1]$ is collinear with, rather than a circulation permutation of, the DNA of the mature Mu-1 bacteriophage. Observation of the position of the inserted Mu defines a point within the gene that has been inactivated (the *lacI* gene for *Flac* and a transfer gene in $F_8[1]$ in these particular instances). These examples illustrate a new, general method for physical gene mapping. The episome with Mu DNA inserted into $F_8[1]$ [i.e., $F_8[1](Mu)$], although derived from a single colony, is heterogeneous in that a self-renatured sample shows a nonhomology loop of length 3.0 kb. This nonhomology loop, which has previously been observed in mature Mu-1 DNA, is due to an inversion.

The temperate bacteriophage, Mu-1, induces mutations in its host, *Escherichia coli* K-12. Both genetic and physical evidence support Taylor's hypothesis that a principal mechanism of gene inactivation is the insertion of Mu DNA into the nucleotide sequence of that particular gene (2-6). Mu insertion can take place at a very large number of sites on the *E. coli* genome.

In the present investigation, we have applied the electron microscope heteroduplex method to study the structure of Mu DNA inserted into bacterial *F'* factors. The results show that inserted (prophage) Mu DNA contains the same sequences in the same relative order as does the DNA of the mature phage; this is to be compared to many other temperate phages, such as the lambdoid phages, where the prophage DNA is circularly permuted relative to the DNA of the mature phage.

The physical position of the inserted Mu on the *F'* DNA defines the position of the gene inactivated. This is the basis, we believe, for a broadly applicable method for physical gene mapping.

MATERIALS AND METHODS

Bacteria and Bacteriophages. The bacterial strains used are listed in Table 1. M 13 phage was a kind gift from Dr. A. Forsheit.

Abbreviations and notation: kb, kilo bases, a unit of length equal to one thousand bases or base-pairs on single- and double-strand nucleic acids, respectively. We use the notation *Flac*(Mu) with Mu in parentheses in the standard way to indicate that Mu is a prophage borne by the DNA molecule *Flac*. The notation *Flac*(*ex* AT 3155) means that the episome *Flac* is carried in the bacterial strain AT 3155 and/or that the DNA molecule has been extracted therefrom. The notation $F_8[1]$ is an identifying number for one of the F_8 episomes we have studied (ref. 1).

Methods. The methods used to isolate closed-circular, plasmid DNAs and to analyze their sequence relations by the electron microscope heteroduplex method were described (1).

Direct Lysis Procedure. A bacterial culture in 10 ml of L broth (14) was grown to a density of about 5×10^8 cells per ml. The cells were collected by centrifugation at 5000 rpm for 10 min in an SS-34 rotor of a Sorvall centrifuge, washed with 50 mM Tris-5 mM EDTA-50 mM NaCl, (pH 8.5) buffer, and suspended again in a spheroplast-forming solution of 1 mg/ml of lysozyme and 100 mg/ml of sucrose in this buffer. After incubation at 37° for 15 min, an aliquot of the solution was diluted 1:20 into 0.5 M NH_4Ac (pH 7.5), containing 100 μ g/ml of cytochrome *c*. 50 μ l of this solution was spread onto a hypophase of 0.25 M NH_4Ac (pH 7.5), and the protein film was picked up, stained, and shadowed (8).

RESULTS

Mu Inserted into *Flac*. *Flac*(Mu) is an episome carried by strain AT 3156. It contains Mu DNA inserted into the *I* gene of the *lac* operon (3). Length measurements on *Flac*(Mu), on the *Flac* from which it was derived, and on Mu DNA are presented in Table 1. It may be seen from the table that the difference in length between *Flac*(Mu) and *Flac* is 33.4 ± 3.5 kb, which is, within experimental error, the same as the observed length of Mu DNA (37.3 ± 1.1 kb). We thus agree with Martuscelli *et al.* (3) that the two *F'* factors differ in length by the length of Mu (although our values for the total lengths are somewhat greater). As already pointed out (3), this relation is evidence for the linear insertion of Mu DNA.

In order to study the sequence relations in *Flac*(Mu), the following heteroduplexes and related samples were prepared and studied in the electron microscope: (a) *Flac*(Mu)/*Flac*; (b) Mu DNA self-renatured; (c) *Flac*(Mu)/Mu; (d) *Flac*(*ex* AT 3155)/ $F\Delta(33-43)$; (e) *Flac*(Mu)/Mu/ $F\Delta(33-43)$.

(a) In the electron microscope, the heteroduplex *Flac*(Mu)/*Flac* consisted of a circular duplex segment of length equal to that of *Flac* plus a single-strand insertion loop of length about equal to that of Mu DNA (Figs. 1 and 2a). This result shows that all of the Mu DNA has been inserted into *Flac* as a single continuous linear structure.

(b) Previous work in this laboratory has shown that the Mu DNA sample used is somewhat heterogeneous (Daniell, E., Abelson, J., Kim, J. P. & Davidson, N., manuscript in preparation; similar observations have been made by H. Delius and E. Bade, personal communication). As depicted

TABLE 1. Properties of bacterial strains and of DNA molecules studied

Strain	Plasmid	Chromosomal genotype	Episomal genotype	Size of episome or phage DNA, $\times 10^{-6}$ daltons	Molecular length, kb	Sources and references
AT 3155	<i>Flac</i>	<i>lac</i> Δ 175 (<i>i</i> ⁻) <i>str</i>	<i>tra</i> ⁺ <i>lacI</i> 78 (<i>i</i> ^s)	96.6 \pm 1.0	146.4	Taylor, A. L. (3)
AT 3156	<i>Flac</i> (Mu)	<i>lac</i> Δ 175 (<i>i</i> ⁻) <i>str</i>	<i>tra</i> ⁺ <i>lacI</i> 78 <i>lacI</i> 176 (<i>i</i> ⁻) <i>Mu</i> ⁺	118.7 \pm 2.1	179.8	Taylor, A. L. (3)
JE 5303	F ₈ [1]	<i>gal</i> ₆ <i>trp</i> <i>arg</i> <i>recA</i> <i>str</i>	<i>tra</i> ⁺ <i>gal</i> ⁺ <i>argG</i> ⁺ <i>nadA</i> ⁺	77.8 \pm 2.5	117.9	Ohtsubo, E. (1)
ND 4	F ₈ [1](Mu)	<i>gal</i> ₆ <i>trp</i> <i>arg</i> <i>recA</i> <i>str</i>	<i>tra</i> ⁻ <i>Mu</i> ⁺ <i>gal</i> ⁺ <i>argG</i> ⁺ <i>nadA</i> ⁺	101.8 \pm 1.2	154.4	Present paper
W 3110(Mu)	—	<i>Mu</i> ⁺	—	24.6 \pm 0.8	37.3	Daniell, E. & Abelson, J. (3, 6)
W 1655	F Δ (33-43)	<i>metB</i> T ₆ ^s (λ)- λ ^r	<i>tra</i> ⁺ ϕ II ^s	55.9 \pm 3.1	84.3	Broda, P. (7)

in Fig. 2*b*, a self-renatured Mu DNA sample contains duplex molecules with split ends, *SE*, and, in some cases, with a substitution loop, *G*, close to the split ends, as well as perfect duplexes. The significance of this heterogeneity is not fully understood. However, its occurrence is fortunate in that it makes it easy to identify the Mu duplex region in the various *Flac*(Mu)/Mu hybrids described below.

(c) The *Flac*(Mu)/Mu heteroduplex consists of a circle with the contour length of *Flac*(Mu). It is mainly single-strand, but it contains a linear duplex region corresponding to the Mu DNA. Sometimes the duplex region exhibits the same split ends and/or small substitution loop features seen in the self-renatured Mu, thus confirming its identification. The structures are schematically depicted in Fig. 2*c*, and an electron micrograph is displayed in Fig. 3. *This result shows that Flac(Mu) contains Mu DNA linearly integrated with its sequences in the same relative order as for the DNA of the mature phage.* By contrast, in a heteroduplex of an F' bearing λ prophage DNA with mature λ DNA, a circular duplex region is seen because the sequences of the prophage DNA are a circular permutation of those of the phage DNA (9).

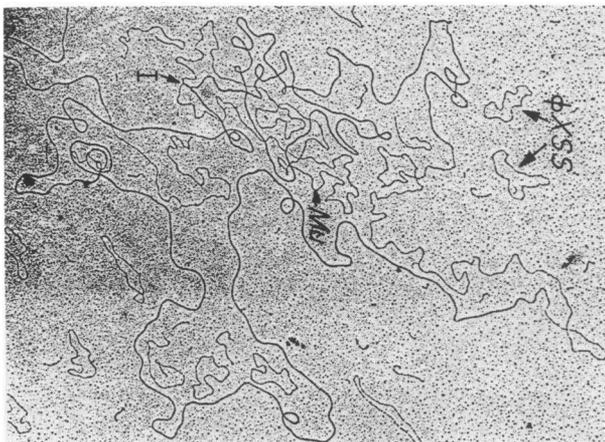


FIG. 1. Electron micrograph of an *Flac*(Mu)/*Flac* heteroduplex. A schematic representation of the structure is shown in Fig. 2*a*. The point at which the single-strand loop of Mu DNA emerges from the duplex is indicated by *I*. Due to some single-strand breaks, a segment of the *Flac* duplex circle at the top of the figure is single-stranded. A point on the single strand of Mu DNA is identified. In this and other micrographs, ϕ X-174 DNA molecules of length 5.25 kb are present for length calibration.

(d) The structures of the episomes *Flac* and *Flac*(Mu) were studied by hybridizing each to the episome F Δ (33-43). We found (1) that F(*ex* W 1485), which is closely related to or identical to the original F of *E. coli* K12, has a length of 94.5 kb, and we have described a coordinate system for specifying the sequences of F. F Δ (33-43) is a deleted F missing the sequence from 32.6 to 42.5 kb (W. M. Anthony, private communication). In heteroduplexes with F or F-prime factors that are not deleted in this region, the readily observable deletion loop provides a reference point for mapping the coordinates of other nonhomology features.

The structure of the heteroduplex *Flac*(*ex* AT 3155)/F Δ (33-43) is shown schematically in Fig. 2*d*. It has the following significant structural features: There is a substitution loop in which the longer arm of length 51.7 kb is the bacterial DNA carried by *Flac* but not present in F Δ (33-43), and the shorter arm of length 3 kb is an F sequence missing in *Flac*. The large deletion loop marked *B* is the F Δ (33-43) deletion and serves as a marker. In addition, there is a small deletion or insertion loop (I-D loop), marked *e*, of length 1.3 kb at coordinate 22.4 kb on the map of F. All these features have been observed in two other *Flacs* studied in this laboratory, *Flac*(*ex* JC 1553) and *Flac*(*ex* DF 3), except that the former doesn't carry loop *e* (M.-T. Hsu, unpublished results). The three *Flacs* are related, in that they are all derived from a common ancestor *Flac* in 200 P.

(e) A micrograph of an *Flac*(Mu)/F Δ (33-43)/Mu di-heteroduplex is shown in Fig. 4, and schematically depicted in Fig. 2*e*. The structure has the features expected from the structure of the *Flac*/F Δ (33-43) heteroduplex discussed in the previous section. There is the large marker loop *B* of F Δ (33-43) at 33 kb and the small I-D loop, *e*, of *Flac* at 22.4 kb. There is a substitution loop with its shorter arm being the F DNA from 0 to 3 kb that is missing in *Flac*. The longer arm of the substitution loop is longer than the corresponding arm of the *Flac* heteroduplex because of the inserted Mu. The most important point is that the position of the Mu DNA duplex region within this arm is readily observed and measured. Its identification is certain, because in different heteroduplexes all three of the self-renatured Mu structures shown in Fig. 2*b* were seen; in the micrograph shown in Fig. 4, split ends are seen. The split ends always occur distal to the marker loop *B*, relative to the junction *b*. Thus, Mu has been inserted with a defined, fixed polarity. We can see in Fig. 2*e* that the Mu DNA insertion in the bacterial genes of *Flac*

occurs at a position 45.7 ± 0.8 kb clockwise from the counter-clockwise junction (identified as *a* in the figure) of F DNA with bacterial DNA in *Flac*. This point of insertion of Mu DNA defines a position within the *lacI* gene. The junction *a* defines the point at which F integrated into *E. coli* K12 to form the Hfr from which *Flac* was derived (1). Thus, a posi-

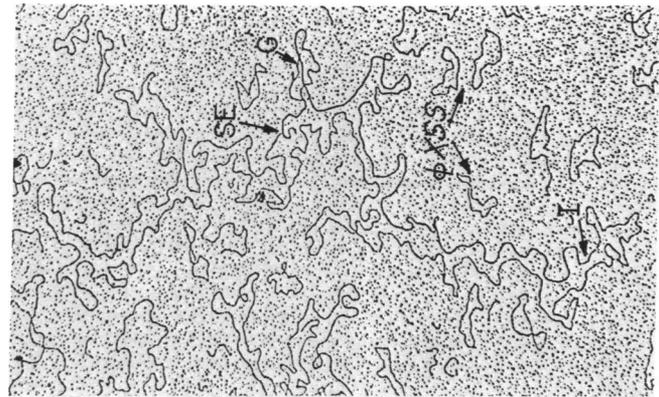


FIG. 3. An *Flac*(Mu)/Mu heteroduplex. Fig. 2c is a schematic structure. The split ends, *SE*, and loop, *G*, are indicated. The other end of the Mu DNA duplex region is indicated by *I* (whereas in Fig. 2c, both ends of the Mu duplex are labeled *I*).

tion within the *lacI* gene in the segment of the *E. coli* K12 chromosome carried by *Flac* has been physically mapped.

Mu Inserted Within a Transfer Gene of *F₈[1]*. *F₈[1]* harbored in strain JE 5303 is a *gal*-bearing, fertile F' factor (10). Its structure has been described. A culture of strain JE 5303 was treated with Mu; surviving lysogens were selected for *gal*⁺ and M 13 resistance (and thus, a probable loss of fertility). The DNA from such a strain (ND 4) had a contour length equal to that of the parental *F₈[1]* plus one Mu length. The structure of the DNA molecule, as determined from a heteroduplex *F₈[1]*(Mu)/*F Δ (33-43)*, is depicted in Fig. 2f. An electron micrograph is shown in Fig. 5. The Mu DNA has been inserted at the coordinate 68 kb on the map of F. This region was identified as one probably containing transfer genes of F (see Fig. 14 of ref. 1), in agreement with the present result that insertion of Mu is correlated with resistance to M 13 phage.

In the *F₈[1]*(Mu)/Mu heteroduplex the same Mu structures are seen as in the *Flac*(Mu)/Mu heteroduplex, and in self-renatured Mu. Thus, in *F₈[1]*(Mu) as in *Flac*(Mu), Mu is integrated with the same linear order of sequences as in the mature phage.

Intracellular Forms of Mu. We have searched for the kinds of closed-circular molecules observable by direct lysis of a cell bearing Mu inserted in an episome. It is our general experience that when a cell bearing a plasmid is subjected to direct lysis and the DNA is spread for electron microscopy as described in *Methods*, it is possible by careful searching of the grids to recognize twisted circular molecules. The molecules are usually so tightly supercoiled that, in many places, the two duplex filaments are twisted around each other and appear as a single thicker filament. Calibration experiments have shown that the contour lengths of these thicker filament regions times 2.4 plus the lengths of the several duplex regions gives the contour length of the DNA molecule observed as a relaxed circle. By direct lysis of an exponentially growing culture of of the strain ND 4, carrying the *F₈[1]*(Mu) episome, we observe two size classes of molecules. The major population of molecules had the length expected for *F₈[1]*(Mu). The minor population has the length expected for Mu DNA. We propose that this is an intracellular form of Mu.

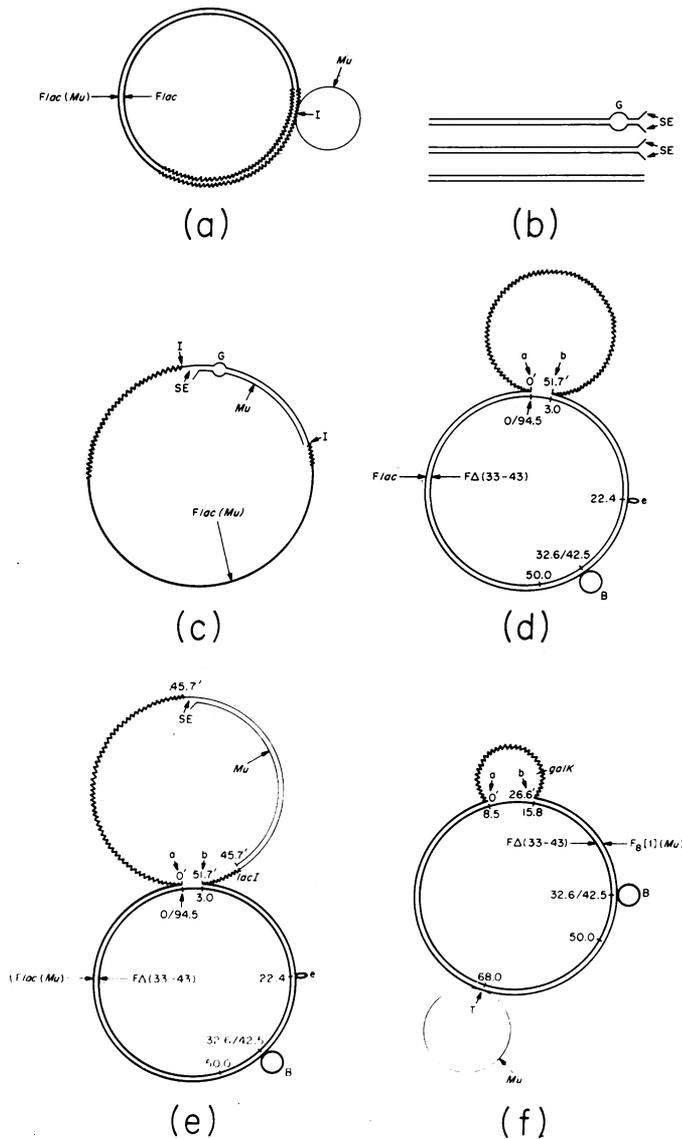


FIG. 2. Schematic representations of heteroduplex structures. (a) The *Flac*(Mu)/*Flac* heteroduplex shown in Fig. 1. *I* is the point at which Mu inserted into *Flac*. (b) Structures seen in self-renatured Mu (Daniell, Abelson, Kim, and Davidson, unpublished); the split ends are indicated by *SE*; the internal non-homology loop of length 3.0 kb by *G*. (c) *Flac*(Mu)/Mu heteroduplex; see also Fig. 3. The split ends, *SE*, and loop, *G*, are indicated. (d) *Flac*/*F Δ (33-43)* heteroduplex. Straight lines are F sequences, sawtooth lines are bacterial DNA sequences; unprimed and primed numbers are coordinates in kb of F and bacterial DNA sequences, respectively. *a* and *b* are the junctions of F and bacterial DNA. Loop *B* is the 9.9-kb deletion of *F Δ (33-43)*; loop *e* is a characteristic of this *Flac*. (e) The *Flac*(Mu)/*F Δ (33-43)*/Mu diheteroduplex (see *d*). The Mu prophage is inserted at coordinate 45.7' kb of the bacterial DNA. (f) *F₈[1]*(Mu)/*F Δ (33-43)* heteroduplex; see also Fig. 5.

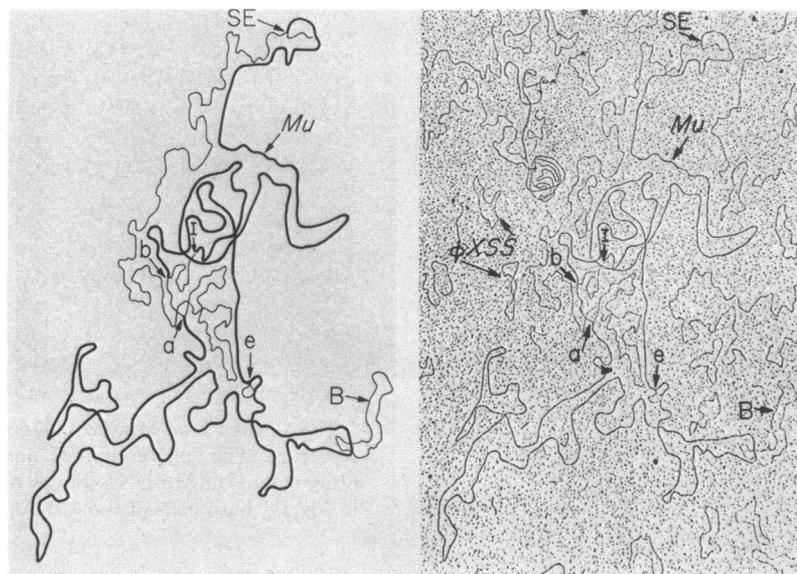


FIG. 4. A *Flac*(Mu)/ $F\Delta(33-43)$ /Mu diheteroduplex, with interpretative tracing. See also Fig. 2e. The point labeled *I* is the same as the point labeled 45.7', *lacI* in Fig. 2e.

Heterogeneity of Mu in $F_8[1]$ (Mu). ND 4 bearing the episome $F_8[1]$ (Mu) was derived from a single colony. In a self-renatured sample of $F_8[1]$ (Mu), perfect circular duplexes were seen. In addition, circular duplexes of the contour length of $F_8[1]$ (Mu), but with a single-substitution loop of length equal to 3.0 kb, are observed (Fig. 6). We believe that this nonhomology region is the internal nonhomology loop *G* of Mu (Fig. 2b).

In heteroduplexes $F_8[1]$ (Mu)/Mu, molecules of the type shown in Fig. 7 are seen at a low frequency. There is a complete single-strand of $F_8[1]$ (Mu) and a complete linear Mu; they are associated by a duplex region of length 3.0 kb at the position of Mu where the nonhomology loop *G* occurs. The interpretation of these findings is that in the prophage in $F_8[1]$ (Mu), the sequence *G* of Mu DNA occurs in two states, one the inverse of the other. It should be noted that this inversion occurs even though the bacteria were derived from a single colony and were *recA*⁻. We have further observed

that the same inversion occurs in mature Mu DNA itself; this is the cause of the loop *G* in self-renatured Mu (Fig. 2b).

DISCUSSION

The present physical study shows that Mu DNA inserted into the bacterial chromosome contains the same sequences in the same order as does the DNA of the mature bacteriophage. The prophage is not circularly permuted. We proposed that, after infection, Mu DNA forms a circular molecule. (Our observation of intracellular circular molecules of the correct length, as described above, supports this view.) The circular molecule then inserts into the bacterial chromosome according to the Campbell model (11), but the insertion point is at the junction of the two ends of the mature linear molecule. It is, of course, also possible that the insertion point is a very short distance, perhaps 10–12 nucleotides, away from the ends. Such a short, circularly-permuted sequence in the prophage might be too short to permit cyclization in a hetero-

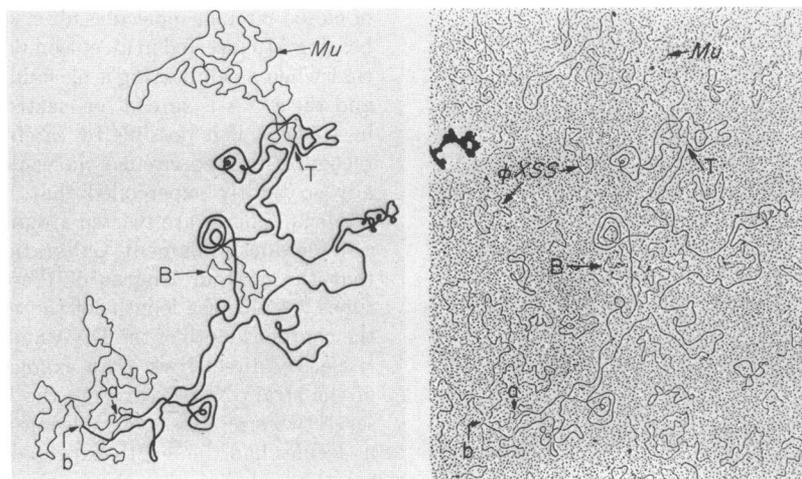


FIG. 5. The $F_8[1]$ (Mu)/ $F\Delta(33-43)$ heteroduplex with tracing. See also Fig. 2f.

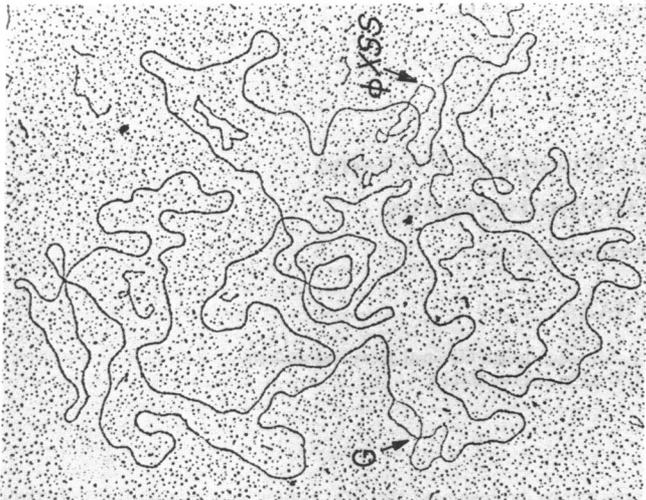


FIG. 6. $F_8[1](Mu)$ self-renatured. The nonhomology loop G is shown.

duplex with the mature phage DNA. [We note that several investigators reported at the Mu Workshop at the Cold Spring Harbor Laboratory in July of 1972, that the genetic maps of Mu phage and prophage are collinear (A. Bukhari, private communication). Thus, the independent genetic and physical studies are in agreement.]

Genetic and/or physical evidence shows that the DNA of the prophages of λ , other lambdoid phages, P 22, etc. are circularly permuted relative to the mature phage DNA molecule (12).

SPO2 and $\phi 105$ are temperate *Bacillus subtilis* bacteriophages. For SPO 2, the genetic map of the prophage is a circular permutation of the mature phage (Truesdell, S., Scibienski, E. & Romig, W. R., private communication); for $\phi 105$, the two genetic maps are collinear (13). L. Chow in this laboratory has provided direct physical evidence that the prophage of SPO 2 is circularly permuted relative to the DNA of the mature phage, by observing a circular structure in prophage-phage heteroduplexes. She interprets the absence of such structures in renatured prophage-phage mixtures for $\phi 105$ to indicate that prophage $\phi 105$ DNA is collinear with that of the mature phage, in agreement with the genetic results.

However, the present observation of a linear duplex structure for Mu DNA prophage-phage heteroduplexes is the first direct physical evidence for a case of lysogeny by insertion without circular permutation.

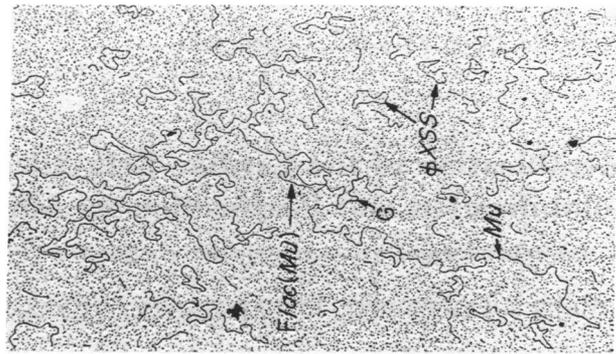


FIG. 7. A linear strand of Mu and a circular strand (actually broken at one point) of $F_8[1](Mu)$ have associated via the duplex segment G only. This and related molecules show that nonhomology loop G in $F_8[1](Mu)$ and in Mu is due to an inversion.

We are grateful to Ellen Daniell and Prof. John Abelson of the University of California at San Diego and to Prof. A. L. Taylor of the University of Colorado Medical School for educating us and stimulating our interest in the properties of Mu-1 bacteriophage DNA. The sample of Mu-1 DNA used in our studies was a generous gift from John Abelson and Ellen Daniell. The first observations in our laboratory of the heterogeneous structure of Mu DNA, as observed in self-renatured samples, were made by J. P. Kim, E. Daniell, and J. Abelson. We appreciate the guidance and advice of E. Ohtsubo throughout this work. This research has been supported by Grant GM 10991 from the NIH. This is Contribution No. 4502 from this Institute.

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