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Isolation of Heterogeneous Circular DNA from Induced Lysogens of Bacteriophage Mu-1

(cesium chloride–ethidium bromide gradients/electron microscopy/heat-inducible mutants)

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ABSTRACT **Covalently-closed circular DNA molecules are formed after induction of phage Mu *cts4* and after infection with phage Mu *cts4*. The circular molecules obtained after induction have a molecular length range from 36.5 to 156.7 kilobases as measured by electron microscopic techniques. These heterogeneous molecules have no consistent correlation to exact multiples of a Mu genome equivalent (37.3 ± 1.2 kilobases). Direct evidence is given that these molecules contain phage Mu DNA that is covalently linked to other DNA sequences.**

Mu-1 is a temperate bacteriophage of *Escherichia coli* K12 that generates stable, random mutations in its host by linearly inserting its DNA into bacterial genes (1–3). The phage genome can be inserted in a clockwise or counter-clockwise orientation with respect to the bacterial chromosome (4), and the genetic and physical maps of prophage and vegetative phage are congruent (4, 5). Since the DNA isolated from mature virions is linear (2), it seemed reasonable to us that the ends of Mu DNA may orient prior to integration by forming a circular DNA molecule. Covalently-closed circular DNA molecules have been observed in Mu lysogens (5) and more specifically in lysogenic bacteria after induction of a heat-inducible mutant prophage (6). Here we further characterize these circular molecules by electron microscopic analysis.

MATERIALS AND METHODS

Bacteria and Bacteriophage. Bacterial strains used in this study are described in Table 1. Mu *cts4* is a heat-inducible mutant of phage Mu isolated in this laboratory by E. Raizen.

Analysis of Intracellular Events. Cells were induced at 45° at a cell density of 1 to 2 × 10^8 cells per ml in T broth (2) supplemented with thymine (4 μg/ml). At 5 min after induction, [*methyl*-^{3}H]thymidine (7 μCi/ml) was added, and 23 min later a 15-ml sample was removed and added to 15 ml of cold TES buffer (0.05 M NaCl, 0.05 M Tris, 0.005 M EDTA, pH 8.0), containing 0.01 M sodium azide. Incorporation of labeled thymidine was linear for 10 min under these conditions. The remainder of the culture was monitored for lysis by measuring decline in turbidity. The DNA was isolated by the Bazaral and Helinski procedure (8), collected, and counted as previously described (2). Fractions were dialyzed for 24 hr against a solution containing 0.8 M NaCl, 0.01 M EDTA, 0.05 M Tris (pH 8.5) and 24 hr against DNA buffer (0.25 M NaCl, 0.01 M EDTA, 0.05 M Tris, pH 8.5) and the absorbancy was determined at 260 nm.

For the experiment in Fig. 1, a culture was grown and induced as above, but it was shifted to 37° at 35 min after induction. Samples were removed at various times and assayed appropriately on L agar (1) for colony forming units and on indicator strain C600 for chloroform-resistant and total phage

Mitomycin Treatment. Cells were grown in T broth supplemented with thymidine (8 μg/ml) and then treated prior to induction with mitomycin C (10 μg/ml) at 33° according to the procedure of Lindqvist and Sinsheimer (9). At the time of induction [*methyl*-^{3}H]thymidine (1 μCi/ml) was added and then at the indicated times 0.5-ml samples were removed and precipitated in 1 ml of cold 10% trichloroacetic acid. Samples were collected onto Whatmann GF/C filters, washed, dried, and counted.

Isolation of Mu cts4 DNA. A Mu *cts4* lysogen was heat induced in 500 ml of L broth at a cell density of 4 to 5 × 10^8 cells per ml. At the time of induction, separately sterilized solutions were added to give a final concentration of 4 mM $MgSO_4$, 0.01% gelatin, and 0.5% glucose. The culture was incubated for 35 min at 45°, and then shifted to 37° until lysis was complete. The phage in clarified lysates were collected by centrifugation and resuspended in Mu buffer (100 mM NaCl, 10 mM $CaCl_2$, 1 mM $CdCl_2$, 40 mM Tris, pH 7.4) without calcium. Phage were further purified by centrifugation in a cesium chloride density gradient (ρ = 1.46 g/cm^3) at 4° in an SW65 rotor for 18 hr at 33,000 rpm. The purified phage were dialyzed against Mu buffer and stored at 4°. Mu *cts4* DNA was extracted and purified as previously described (2).

TABLE 1. *Bacterial strains*

Strain no.	Genotype*					Mating type
AT3088	*thyA16*	*thyR4*	*thi-1*			Hfr Hayes
AT4510	*thyA16*	*thyR4*	*thi-1*	Mu *cts4*		Hfr Hayes
AT4517	*thyA16*	*thyR4*	*thi-1*	Mu *cts4*		F$^-$
AT4530	*thyA16*	*thyR4*	*thi-1*	*pon-8*	Mu *cts4*	F$^-$
AT4518	*thyA16*	*thyR4*	*thi-1*			F$^-$
AT4520	*thyA2*	*his-1*	*lysA10*	*metB1*	Mu *cts4*	F$^-$
C600	*thr-1*	*leu-6*	*thi-1*			F$^-$

* Genetic symbols are those described by Taylor and Trotter (7), except for *thyR* which denotes mutants able to grow on low levels (4 μg/ml) of thymine. The *pon-8* mutation confers simultaneous resistance to phages Mu and P1. The chromosomal location of prophage is unknown in all lysogens of Mu *cts4* listed above.

Abbreviations: PFU, plaque-forming units; CCC, covalently closed circular.

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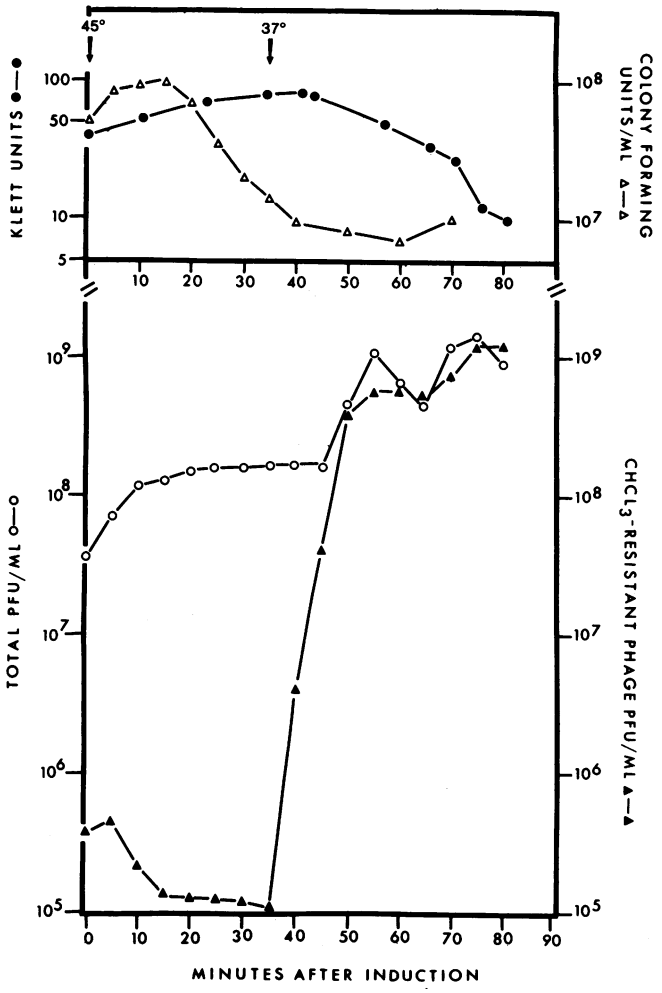

FIG. 1. Intracellular events which take place after heat induction of the Mu *cts4* lysogen, AT4517.

Electron Microscopy. Preparation of heteroduplex DNA and mounting of native and heteroduplex DNAs by the formamide method were done essentially as described by Sharp *et al.* (10). Length measurements were made from tracings of photographic negatives enlarged with a Scherr-Tumico optical comparator.

RESULTS

Heat Induction of Mu cts4 Lysogens. The intracellular events which occur after heat induction of prophage in strain AT4517 are shown in Fig. 1. Cell viability begins to decline after 10–15 min at the inducing temperature of 45°. Mature intracellular phage, released by chloroform, appears from 35 min onward and the first extracellular phage is seen at 45 min. The onset of lysis occurs routinely 40–48 min after induction.

To measure the synthesis of vegetative Mu DNA, we used mitomycin C to selectively suppress host DNA synthesis (9). Cultures of lysogenic and nonlysogenic control bacteria were grown at 45° with or without mitomycin C pretreatment. As shown in Fig. 2*A*, the treated cells incorporated labeled thymidine to a lesser extent than untreated controls, indicating that host DNA synthesis is being reduced. Net synthesis of phage DNA first becomes evident 20–30 min after induction and continues to rise throughout the lytic cycle. The eclipse period in mitomycin-treated cultures is extended by approximately

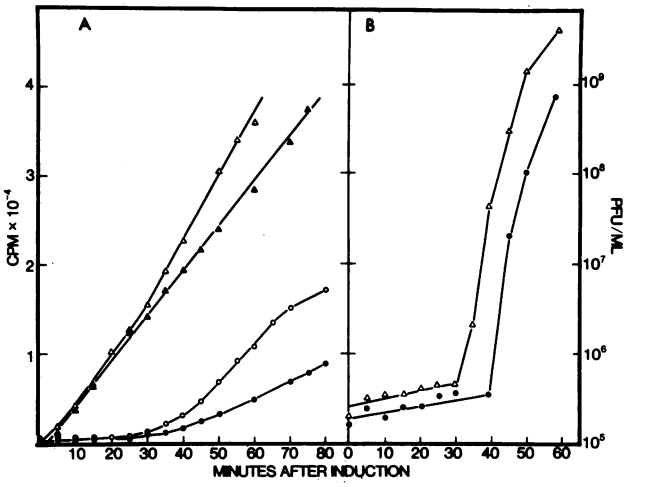

FIG. 2. The effect of mitomycin C on (*A*) bacterial and phage DNA synthesis as measured by incorporation of [^{3}H]thymidine and (*B*) the time of appearance of chloroform-resistant intracellular phage. Strain AT4530 (Mu *cts4*), pretreated (O) and untreated (△) with mitomycin. Strain AT4518 (nonlysogenic), pretreated (●) and untreated (▲) with mitomycin. (The numbers on the left ordinate have been multiplied by 10^{-4}.)

10 min (Fig. 2*B*) and the final yield of phage plaque-forming units (PFU) (not shown) is reduced by about 50%.

Covalently-Closed Circular DNA in Heat-Induced Lysogens. The DNA from a heat-induced culture of strain AT4510 was labeled and purified in a cesium chloride–ethidium bromide density gradient. A typical profile of the labeled DNA is

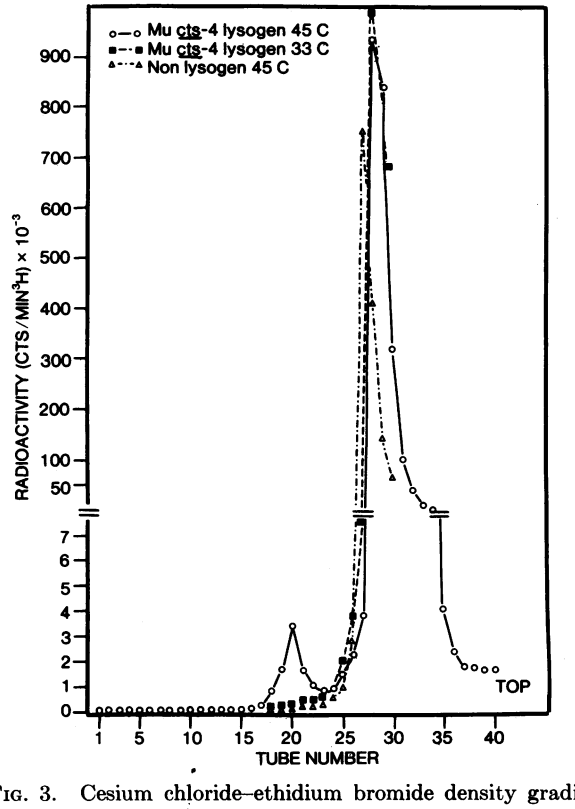

FIG. 3. Cesium chloride–ethidium bromide density gradient profile of labeled DNA isolated 28 min after induction. Strain AT4510 (Mu *cts4*), induced at 45° (O) and uninduced at 33° (■). Strain AT3088 (non-lysogenic), grown at 45° (△). (The numbers on the ordinate have been multiplied by 10^{-3}.).

TABLE 2. *Lengths of individual covalently-closed circular DNA molecules*

Molecular length range, kb*								
0–34†	35–50	51–66	67–82	83–98	99–114	115–130	131–146	147–162
	36.5	51.2	67.0	83.3	98.5	116.2	133.8	156.7
	37.0	51.7	67.7	85.3	99.4	119.7	136.1	
	37.1	53.6	69.0	85.3	100.5	120.9	136.8	
	37.6	55.2	71.1	88.0	101.4	123.3		
	39.2	56.4	71.7	88.0	107.6			
	40.3	57.0	72.7	88.5	110.0			
	40.9	57.6	73.3	88.5	110.9			
	44.7	58.3	75.2	92.4	112.1			
	46.7	59.2	75.8	93.2	113.6			
	50.0	61.0	77.3	93.5				
		63.0	81.4	98.2				
		65.3	82.0					
		65.5						
	37.3		*74.6*		*111.9*			*149.2*

Length measurements were calibrated against circular double-stranded phage PM2 DNA, of molecular weight 6×10^6 (15) and length 9.1 kb. Values in italics are the lengths predicted for circles containing integral multiples (1 to 4) of a Mu genome (2, 14).

* One kilobase (kb) corresponds to 1000 bases or base pairs.

† Molecules of PM2 size are excluded from this column.

shown in Fig. 3. The small peak of higher density corresponds to a position in the gradient where covalently-closed circular (CCC) DNA molecules would band (11). The amount of labeled DNA in this peak represents 0.3–0.6% of the counts present in the bulk chromosomal DNA peak. Kiger and Sinsheimer (12) also reported that less than 1% of incorporated label appears in CCC DNA after heat induction of phage lambda. However, we found that the CCC DNA band shown in Fig. 3 actually contains 12% of the A_{260} which is present in bulk DNA. Although this unexpected result could be explained in part by our labeling procedure, it could be also that unlabeled sequences derived from host DNA may contribute to the pool of CCC DNA molecules. To test this possibility, [^{3}H]thymidine was added to cultures several generations before induction. Under these conditions, the percent radioactivity of the CCC DNA peak increased to a value approaching that expected from the A_{260} measurements. Thus, it appears that bacterial DNA sequences not only participate in the formation of circular molecules but also comprise a major proportion of CCC DNA found after induction of prophage.

Several control experiments show that the appearance of these CCC molecules is a specific consequence of Mu *cts4* prophage induction. First, no CCC DNA is produced in strain AT4510 grown at the noninducing temperature of 33°, nor is it produced in strain AT3088 (the nonlysogenic progenitor of AT4510) grown at 45° (Fig. 3). Second, we considered the possibility that induction of Mu might stimulate excision of covalently closed sex factor DNA in the Hfr strain AT4510. An F$^-$ derivative of AT4510 (designated AT4517) produced similar amounts of CCC DNA as AT4510, indicating that sex factor DNA is not involved. Third, an independent Mu *cts4* lysogen (AT4520) was isolated to test the effects of a changed prophage location and a different genetic background on circle formation. Upon induction at 45°, this lysogen produced CCC DNA in a manner similar to AT4510.

We have also observed a peak of CCC DNA similar to the one shown in Fig. 3 after infection of the Mu-sensitive strain AT4518 with phage Mu *cts* at 37°. Thus, the formation of circular DNA is not limited to prophage induction, but appears to be a general characteristic of phage Mu development.

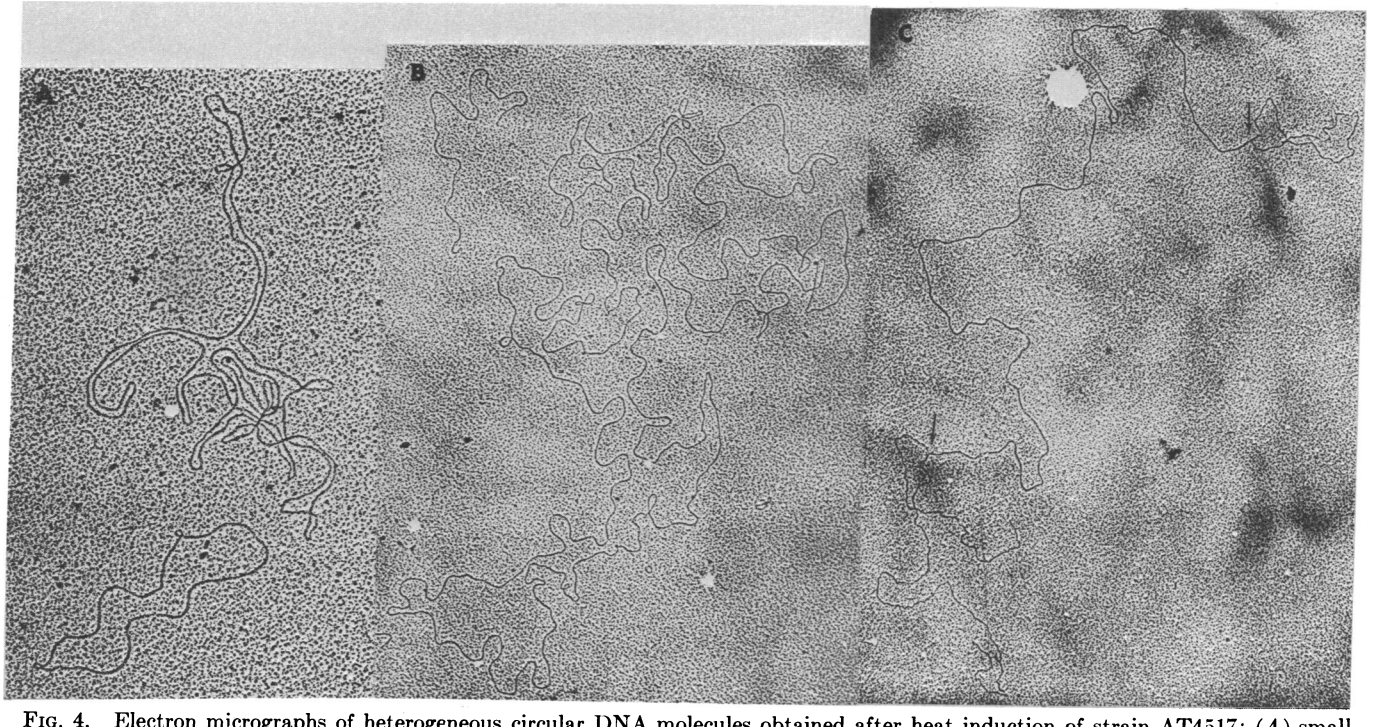

FIG. 4. Electron micrographs of heterogeneous circular DNA molecules obtained after heat induction of strain AT4517: (*A*) small circular molecule, 37.1 kb in length and one molecule of standard PM2 DNA; (*B*) larger circle, 120.9 kb in length; (*C*) single strand containing an inverted repeat sequence 38.0 kb in length, from denatured and self-annealed circular DNA. *Arrows* indicate the ends of the internally renatured repeat sequence.

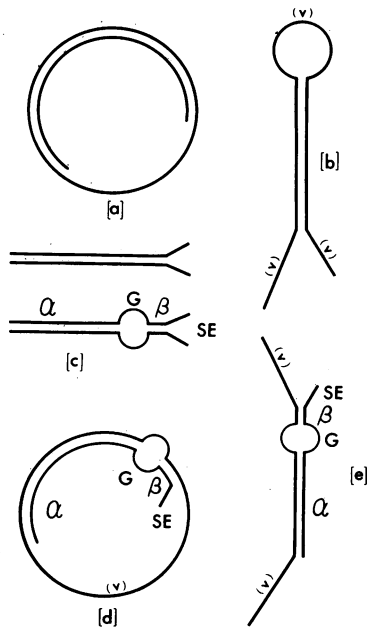

FIG. 5. Schematic representation of duplex structures observed in self-renatured circular DNA from an induced Mu lysogen: [*a*] intact circle, partially renatured; [*b*] broken single strand containing an inverted repeat sequence of length corresponding to Mu DNA (see also Fig. 4*c*). Heteroduplexes observed between circular DNA and Mu *cts4* DNA: [*c*] heteroduplexes of mature Mu DNA, showing single-stranded split ends (*SE*) and bubble (*G*); [*d*] heteroduplex of mature Mu DNA with a larger intact circle (see also Fig. 6); [*e*] heteroduplex of mature Mu DNA with a longer broken strand. The single-stranded regions marked (*V*) are variable in length.

Molecular Length of CCC DNA Molecules. Fig. 4*A* and *B* shows two examples of relaxed circular molecules from a heat-induced lysogen. The molecules isolated here were exceedingly variable in size and the lengths of 63 circles photographed at random ranged from 36.5 to 156.7 kb (Table 2). Most of the values in this sample do not correspond to integral multiples of the length of mature Mu DNA (37.3 ± 1.2 kb) and in addition, there is an apparent preponderance of molecules in the size range of 55–74 kb.

Mu DNA in the Circular Molecules. Purified circular DNA was denatured, self-annealed, and mounted for electron microscopy. We observed a total of 46 self-annealed structure, 40 of which were double-stranded circles of variable length within the range reported in Table 2. We observed one partially double-stranded circle (Fig. 5*a*) which may represent either a heteroduplex between different sized circles having a common sequence or a homoduplex between an intact and a broken complementary strand. Of the self-annealed structures, five had a hairpin configuration (Fig. 4*C* and 5*b*), indicating that some of the molecules contain an inverted repeat sequence that enables the strands to renature with themselves. The lengths of the double-stranded repeat segments of three hairpins in which two single-stranded tails were clearly visible were 27.3, 30.8, and 38.0 kb. Lengths of the single-stranded loops connecting the inverted sequences were variable and ranged from 7.9 to 14.7 kb. Although somewhat variable in this small sample, the lengths obtained for the repeat sequence are consistent with the interpretation that some of the circular DNA molecules contain two Mu genomes inserted in opposite orientation. González and Taylor (manuscript in preparation) have found that *Flac* DNA containing two Mu prophages in opposite orientation similarly produces hairpin structures upon denaturation and self-renaturation.

To obtain more direct evidence for the presence of Mu DNA in the circles, we prepared heteroduplexes between circular DNA and mature linear Mu *cts4* DNA. Of 33 duplex structures seen in this experiment, 17 were typical heteroduplexes of mature Mu DNA (5, 13, 14, J. Goddard and A. L. Taylor, unpublished data on Mu *cts4*). They are characterized by single-stranded split ends (*SE*) and, in 12 molecules, by a single-stranded bubble (*G*) as well; the duplex segments to the left and right of the *G* bubble are designated α and β, respectively (Fig. 5*c*). Heteroduplexes between mature Mu DNA and circular DNA were unmistakably identified by the presence of *G* bubbles, as shown in Fig. 6. We observed two heteroduplexes between Mu DNA and intact circular DNA (Fig. 5*d*) and seven heteroduplexes between Mu DNA and broken linear strands derived from circular molecules (Fig. 5*e*). The lengths of the α, *G*, β, and *SE* segments in these structures were identical to those of heteroduplex Mu DNA in the same preparation. We believe that this is direct evidence that the CCC DNA molecules formed after heat induction contain phage Mu DNA covalently linked to other DNA, probably of bacterial origin. A more detailed description to these heteroduplex structures will be presented elsewhere.

DISCUSSION

We have established that there is a circular DNA form produced specifically after induction of Mu *cts4* prophage and after infection with phage Mu *cts4*. Other temperate phages, e.g., lambda and P22, are known to produce circular DNA of a phage genome size in the course of viral development (16, 17). Multiple-lengthed circles corresponding to dimers, trimers, and higher concatemers of φX174 DNA have been described as well (18). The heterogeneous circular molecules isolated after induction of Mu differ from other systems, however, as they reveal no consistent correlation to integral multiples of the phage genome. While we known of no other phage exhibiting this property, Radloff described a heterogeneous distribution of DNA circles in HeLa cells (11).

The smallest circles in the heterogeneous population obtained after induction have a length identical to that of mature Mu DNA and thus, may be circular monomers of the phage genome. Hsu and Davidson (5) also reported circles of one Mu length in uninduced normal lysogens. We postulate that these circles are generated by precise excision of Mu from the bacterial genome and further that these may represent an integrative form of Mu DNA. This view is in accord with all available physical and genetic evidence which shows that lysogenization involves insertion of Mu DNA without concomitant insertion of other large DNA sequences (2, 3, 5).

Our data show that the majority of circles found at 28 min after induction are longer than a Mu genome and that at least some of the circles are 'hybrid' molecules which contain Mu DNA in association with other DNA sequences. W. Schröder, E. Bade, and H. Delius (personal communication) have shown by means of DNA–DNA hybridization that sequences complementary to both phage and bacterial DNA are present in the population of circular molecules formed after infection of bacteria with wild-type phage Mu.

We propose two possible mechanisms for the generation of heterogeneous 'hybrid' molecules. They could arise by simultaneous excision and circularization of prophage DNA to-

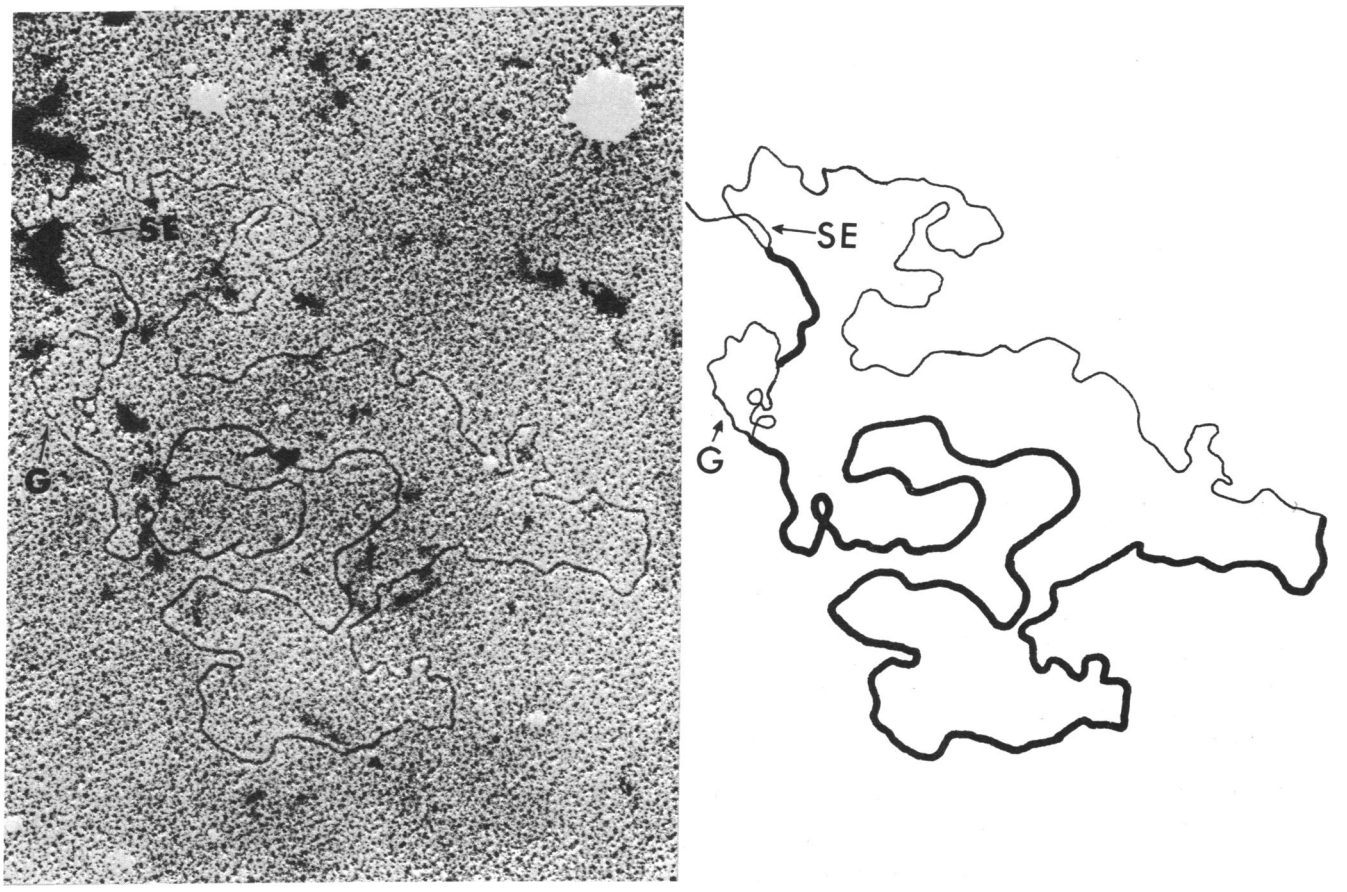

FIG. 6. Heteroduplex of Mu and circular DNA, with an interpretative tracing showing the split end (*SE*) and bubble (*G*).

gether with variable amounts of host DNA adjacent to the prophage. The high proportion of shorter molecules in the total population (Table 2) may represent such initial excision products. Larger circular molecules could be generated by recombination events between homologous regions of two smaller molecules. Our interpretation that some molecules contain two sequences in opposite polarity also suggests that additional phage genomes can be inserted into the initial excision products to generate larger circles.

An alternative mechanism would be for Mu to integrate into pre-existing circular molecules derived *exclusively* from host DNA under the influence of Mu determined enzymes. Our A_{260} measurements indicate that a major portion of the CCC DNA is host DNA and it is therefore probable that many of the individual molecules contain pure bacterial DNA. This may help to explain why heteroduplexes between circular DNA and linear Mu DNA were seen so infrequently in the electron microscope.

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1. Taylor, A. L. (1963) *Proc. Nat. Acad. Sci. USA* **50,** 1043–1051.
2. Martuscelli, J., Taylor, A. L., Cummings, D. J., Chapman V. A., DeLong, S. S. & Canedo, L. (1971) *J. Virol.* **8,** 551–563.
3. Bukhari, A. & Zipser, D. (1972) *Nature New Biol.* **236,** 240–243.
4. Abelson, J., Boram, W., Bukhari, A. I., Faelen, M., Howe, M., Metlay, M., Taylor, A. L., Toussaint, A., Van De Putte, P., Westmaas, G. C. & Wijffelman, C. A. (1973) *Virology* **54,** 90–92.
5. Hsu, M. T. & Davidson, N. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 2823–2827.
6. Waggoner, B. T. & Taylor, A. L. (1973) *Abstracts of Annual Meeting, American Society for Microbiology,* p.218.
7. Taylor, A. L. & Trotter, C. D. (1972) *Bacteriol. Rev.* **36,** 504–524.
8. Bazaral, M. & Helinski, D. R. (1968) *J. Mol. Biol.* **36,** 185–194.
9. Lindqvist, B. H. & Sinsheimer, R. L. (1967) *J. Mol. Biol.* **30,** 69–80.
10. Sharp, P., Hsu, M. T., Ohtsubo, E. & Davison, N. (1972) *J. Mol. Biol.* **71,** 471–497.
11. Radloff, R., Bauer, W. & Vinograd, J. (1967) *Proc. Nat. Acad. Sci. USA* **57,** 1514–1521.
12. Kiger, J. A. & Sinsheimer, R. L. (1969) *J. Mol. Biol.* **40,** 467–490.
13. Bade, E. G. (1972) *J. Virol.* **10,** 1205–1207.
14. Daniell, E., Abelson, J., Kim, J. S. & Davidson, N. (1973) *Virology* **51,** 237–239.
15. Espejo, R. T., Canelo, E. S. & Sinsheimer, R. L. (1969) *Proc. Nat. Acad. Sci. USA* **63,** 1164–1168.
16. MacHattie, L. A. & Thomas, C. A. (1964) *Science* **144,** 1142–1144.
17. Rhoades, M. & Thomas, C. A. (1968) *J. Mol. Biol.* **37,** 41–61.
18. Rush, M. G., Kleinschmidt, A. K., Hellmann, W. & Warner, R. C. (1967) *Proc. Nat. Acad. Sci. USA* **58,** 1676–1683.