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Structure and Restriction Enzyme Maps of the Circularly Permuted DNA of Staphylococcal Bacteriophage ϕ11

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One restriction enzyme map of *Staphylococcus aureus* bacteriophage ϕ11 DNA was established by reciprocal double digestions with the enzymes *Eco*RI, *Hae*III, and *Kpn*I. The sequential order of the *Eco*RI fragments was thereafter established by a novel approach involving blotting of DNA partially cleaved with *Eco*RI and then probing the blots with nick-translated terminal fragments. A circular map of the ϕ11 DNA was established, and the phage genome was circularly permuted based on the failure to end label mature viral DNA, restriction maps of replicating DNA, and finally, homoduplex analysis in the electron microscope. A restriction enzyme map of the prophage form of ϕ11 DNA was obtained by analysis of chromosomal DNA from a lysogenic strain.

Bacteriophage ϕ11 is a group B phage first detected as a prophage of *Staphylococcus aureus* NCTC 8325 (11, 12). This phage is of genetic importance since it appears to be required to establish competence for transformation and transfection in *S. aureus* (15). Genetic experiments suggested that expression of an early viral gene product is required to establish competence (16). This conclusion was recently challenged by Thompson and Pattee (20), who suggested that the competence-inducing factor resided in the phage particle. A circular genetic map of phage ϕ11 was established with the aid of *sus* mutants of the phage (5). Several temperature-sensitive (*ts*) mutants, including the only mutant (ϕ11 *ts*31) exhibiting a temperature-sensitive induction of competence, could be located on the circular map (16).

The structural properties of the phage and of the viral DNA were investigated by Brown et al. (3). They observed molecular weights of 66.7 $\times$ 10^6 for the phage and 30.6 $\times$ 10^6 (46.0 kilobase pairs [kbp]) for the single molecule of DNA in the phage head. The DNA is linear and double stranded and contains no apparent single-stranded (ss) terminal redundancy.

This study reports a more detailed analysis of phage ϕ11 DNA. Restriction enzyme maps of mature phage DNA, prophage DNA, and replicative DNA circles have been established. It is concluded that the mature DNA is circularly permuted. A novel approach to determining the sequential arrangement of restriction enzyme fragments in circular permuted DNA was used to establish the restriction enzyme maps.

MATERIALS AND METHODS

Phage and bacterial strain. Phage ϕ11 was propagated in *S. aureus* 8325-4 as described previously (15). Bacterial cells were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.).

Preparation of DNA. Adenovirus type 2 DNA was prepared as described previously (21). Phage ϕ11 stocks were derived from single-plaque isolates. The phages were concentrated by centrifugation in a Spinco SW27 rotor at 25,000 rpm for 90 min. The pellet was suspended, and the phages were purified on preformed CsCl gradients (1.35 to 1.55 g/cm^3) in 10 mM Tris-hydrochloride (pH 7.5)-10 mM $MgCl_2$. The phage band was collected and dialyzed against the same buffer. Phage DNA was extracted by incubating the phage preparation for 2 h at 37°C in the presence of 0.5% sodium dodecyl sulfate and 0.5 mg of proteinase K per ml followed by phenol extraction and dialysis against TE buffer (10 mM Tris-hydrochloride, pH 7.5, 1 mM EDTA). The covalently closed circular form of phage ϕ11 DNA was prepared from cells infected at a multiplicity of 1. At 20 min after infection 10 μg of chloramphenicol per ml was added. The cells were chilled on ice, and covalently closed circular DNA was prepared as described previously (7).

Electron microscopy. Linear DNA prepared from phage ϕ11 particles was analyzed by the aqueous method at pH 7.5, and homoduplex analysis of the mature phage DNA was performed as described in reference 4.

Restriction endonucleases. Digestions with restriction endonucleases were performed at 37°C for 1 to 18 h in a buffer containing 6 mM Tris-hydrochloride (pH 7.5)-$MgCl_2$-mercaptoethanol, with the addition of salts as recommended by the manufacturers. *Sma*I digestion was made in a buffer containing 30 mM Tris-hydrochloride (pH 9.0)-3 mM $MgCl_2$-20 mM KCl at 30°C for 30 min. Before loading onto agarose gels, EDTA was added to 5 mM and Ficoll (Pharmacia Fine Chemicals Ltd., Uppsala, Sweden) was added to 2%.

Agarose gel electrophoresis. DNA was electrophoresed in vertical slab gels containing various agarose concentrations in 0.5× TEB (1× TEB is 89 mM Tris-hydrochloride [pH 8.3], 0.25 mM EDTA, 99 mM boric acid). Gels were stained with ethidium bromide (1 μg/ml).

Recleavage of DNA fragments from agarose

gels. DNA fragments of 0 to 4 megadaltons in size were best eluted by squeezing the piece of agarose suspended in TE buffer through a syringe and allowing the DNA to diffuse out of the gel at 37°C overnight. The agarose was removed by centrifugation, and the supernatant was passed through a DEAE column. The DNA was eluted with 0.6 M NaCl, precipitated with ethanol, and dissolved in TE buffer. DNA obtained by this procedure was easily digested with restriction enzymes. Larger fragments were prepared by electrophoresis in a plastic cylinder, in which the DNA (before entering the hydroxylapatite) was passed through both a 1% agarose plug and a Sephadex G-50 column. The DNA was eluted with 1 M phosphate buffer, pH 6.8, and diluted three times in water. This solution was applied to a DEAE-cellulose column and eluted in 0.6 M NaCl precipitated in ethanol and dissolved in TE buffer. This method gave a final recovery of 30 to 40% of the DNA, which was readily digested with other restriction enzymes.

Transfer of DNA to nitrocellulose filters and hybridization. Native DNA in agarose slab gels was denatured in situ and transferred by blotting onto nitrocellulose filters as described by Southern (18). The DNA used as the probe for hybridization was nick translated in vitro with [α-^{32}P]dCTP by the procedure of Rigby et al. (14). Baked filters were used for hybridization as described by Botchan et al. (2). After incubation at 65°C for 18 to 24 h with the ^{32}P-labeled probe, the filters were washed three times in 2× SSC (1× SSC is 0.15 M NaCl plus 0.05 M sodium citrate) with 0.5% sodium dodecyl sulfate and three times in 2× SSC, dried, and analyzed by autoradiography.

RESULTS

Size of mature phage ϕ11 DNA. DNA prepared from phage ϕ11 particles was studied in an electron microscope, using the aqueous spreading method (4). The contour length measurements of linear ϕ11 DNA gave a molecular weight of $28.7 \times 10^6 \pm 3\%$ (43.4 kbp; Table 1), which is slightly smaller than the values determined by Brown et al. (3). They obtained molecular weights of 32.7×10^6 and 30.6×10^6, respectively, by sedimentation in an analytical ultracentrifuge and contour length measurements. A size of 42.8 kbp was obtained when the sizes of the 15 *Eco*RI-generated fragments were added (Table 2).

Cleavage of phage ϕ11 DNA by various restriction endonucleases. On the basis of the preliminary cleavage patterns, three enzymes, *Kpn*I, *Hae*III, and *Eco*RI, were chosen for detailed analysis. Among other enzymes analyzed, *Bam*HI and *Sma*I obviously lack cleavage sites in the ϕ11 genome. The fragment pattern produced by *Eco*RI, *Hae*III, and *Kpn*I cleavage of ϕ11 DNA is shown in Fig. 1 and Table 2. *Eco*RI produces 15 fragments ranging in size from 9.4 to 0.2 kbp. Fig. 1B shows a 2.2% agarose gel in which the three smallest *Eco*RI fragments (200 to 245 bp) may be detected. Fragments *Eco*RI-F and *Eco*RI-G form a double band which could be resolved on analytical gels. They can be distinguished because *Eco*RI-F has a cleavage site for *Hae*III. *Hae*III produces five fragments and *Kpn*I produces two fragments of phage ϕ11 DNA.

Double digestions. Figure 1 also shows the pattern of the double digests of ϕ11 DNA. Table 2 also summarizes the sizes and designations of all fragments from the double digests. The combinations used were *Eco*RI-*Hae*III, *Hae*III-*Kpn*I, and *Kpn*I-*Eco*RI. In the *Kpn*I-*Eco*RI double digest two *Eco*RI fragments disappear, *Eco*RI-C and *Eco*RI-H. Likewise, two fragments disappear in the *Hae*III-*Kpn*I double digest, *Hae*III-A and *Hae*III-B. Instead, two new large fragments are generated (*Hae*III-A^{K1} and *Hae*III-B^{K1}) which are more separated than *Hae*III-A and *Hae*III-B. This indicates two cleavage sites for *Kpn*I in ϕ11 DNA. Since the prepared DNA is linear, we expected a third *Kpn*I fragment if two sites were available, but no such fragment could be identified. These results suggest that the linear genome is circularly permuted.

The *Eco*RI-*Hae*III double digest eliminates *Eco*RI-C and *Eco*RI-E as shown in Fig. 1. There also appears to be a reduction in size of the *Eco*RI-I and *Eco*RI-B fragments after *Eco*RI-*Hae*III double digestion compared with the *Eco*RI pattern (Fig. 1). The double band corresponding to *Eco*RI-F and *Eco*RI-G also migrates differently after double digestion.

To establish a more detailed relationship between the enzyme restriction fragments, they were isolated from *Eco*RI, *Hae*III, and *Kpn*I digests and redigested with other enzymes. The *Eco*RI fragments were digested with *Hae*III or *Kpn*I, and the products were compared with *Eco*RI-*Hae*III and *Eco*RI-*Kpn*I double digests, respectively. The results in Table 3 show that the *Eco*RI-C fragment is cleaved by *Hae*III, giving a fragment (*Eco*RI-C^{H1}) in the same size range as *Eco*RI-F and -G and another (*Eco*RI-C^{H2}) slightly smaller than *Eco*RI-I, whereas *Eco*RI-F is eliminated by *Hae*III. Thus, the *Eco*RI-B, -C, -E, -F, and -I fragments contain cleavage sites for the *Hae*III enzyme.

When the *Eco*RI-C fragment was cleaved by *Kpn*I, two fragments were generated (*Eco*RI-C^{K1} and *Eco*RI-C^{K2}), 4.6 and 0.38 kbp in size,

TABLE 1. *Contour length of phage ϕ11*

DNA	Mol wt	No. of molecules measured	Marker (mol wt)
ϕ11	28.8×10^6 (± 1.2)	38	ϕX174 RFII (3.55×10^6)
ϕ11	28.6×10^6 (± 0.7)	35	PM2 (6.58×10^6)

TABLE 2. *Size of phage ϕ11 DNA fragments produced by single and double digestions with restriction endonucleases*

Fragment	Single digests			Double digests					
	Size (kbp)			*Eco*RI + *Hae*III		*Eco*RI + *Kpn*I		*Hae*III + *Kpn*I	
	*Eco*RI	*Hae*III	*Kpn*I	*Eco*RI fragment	kbp	*Eco*RI fragment	kbp	*Hae*III fragment	kbp
A	9.4	20.4	27.1	A	9.4	A	9.4	A^{K1}	19.0
B	5.4	13.8	15.8	B^{H1}	5.25	B	5.4	B^{K1}	11.2
C	5.0	4.3		D	3.7	C^{K1}	4.6	C	4.35
D	3.7	3.25		G	3.2	D	3.7	D	3.2
E	3.4	1.1		C^{H1}	3.1	E	3.4	B^{K2}	2.7
F	3.25			H	2.75	F	3.25	A^{K2}	1.4
G	3.2			E^{H1}	2.45	G	3.2	E	1.1
H	2.75			C^{H2}	1.9	J	1.95		
I	1.95			F^{H1}	1.85	H^{K1}	1.9		
J	1.7			J	1.7	J	1.7		
K	1.3			F^{H2}	1.4	K	1.3		
L	1.1			I^{H1}	1.4	L	1.1		
M	0.245			K	1.3	H^{K2}	0.85		
N	0.225			L	1.1	C^{K2}	0.375		
O	0.20			E^{H2}	0.95	M	0.245		
				I^{H2}	0.57	N	0.225		
				M	0.245	O	0.20		
				N	0.225				
				O	0.20				
				B^{H2}	0.14				

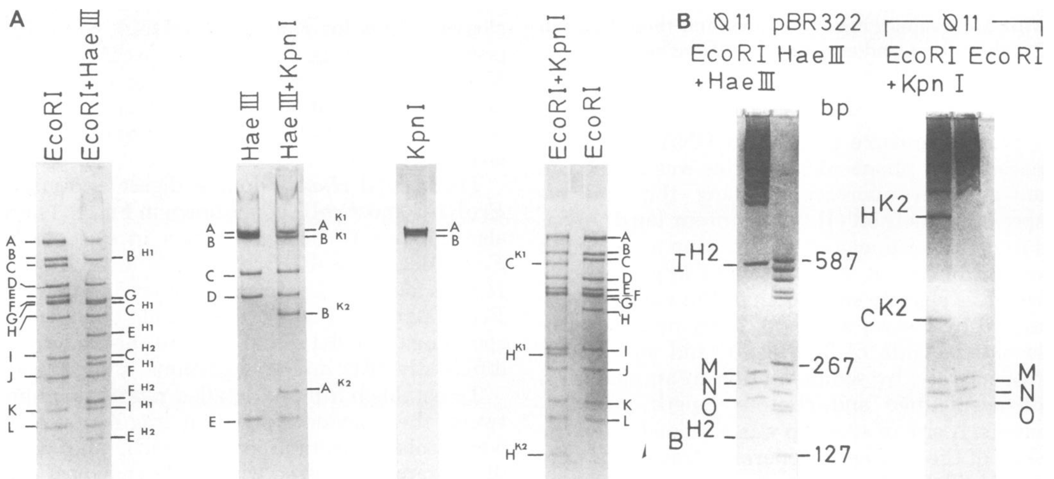


FIG. 1. *Agarose gel electrophoresis of restriction enzyme fragments of phage ϕ11 DNA. Single digestions were carried out with EcoRI, HaeIII, and KpnI, respectively, and double digestions were carried out with EcoRI + HaeIII, HaeIII + KpnI, and EcoRI + KpnI, respectively. The digests were analyzed on a 1% agarose gel (A) with adenovirus type 2 DNA cleaved with SmaI, EcoRI, and BamHI as size markers (not shown) and on a 2.2% agarose gel (B) with pBR322 HaeIII fragments as size markers.*

respectively. The *Eco*RI digestion of *Kpn*I-A and -B reveal that the 4.6-kbp fragment (*Eco*RI-C^{K1}) originates on *Kpn*I-A and the 0.38-kbp fragment (*Eco*RI-C^{K2}) originates on *Kpn*I-B (Table 4). Two fragments (*Eco* RI-H^{K1} and *Eco*RI-H^{K2}, 1.9 and 0.85 kbp) were obtained by *Kpn*I cleavage of *Eco*RI-H. The reverse experiment, cleaving *Kpn*I-A and -B by *Eco*RI, revealed that the 1.9-kbp fragment (*Eco*RI-H^{K1}) is derived from *Kpn*I-A and the 0.85-kbp fragment (*Eco*RI-H^{K2}) is derived from *Kpn*I-B. Thus, the cleavage sites of *Kpn*I are located on the *Eco*RI-C and *Eco*RI-H fragments.

The results of digestion of isolated *Hae*III

fragments are shown in Table 5. The cleavage products were compared with the double digests *Eco*RI-*Hae*III and *Hae*III-*Kpn*I. Two new fragments are produced by *Eco*RI digestion of *Hae*III-A, one 0.57-kbp fragment (*Eco*RI-I^{H2}) corresponding to the smaller of the two fragments produced by *Hae*III digestion of *Eco*RI-I (Table 3) and another 5.25-kbp fragment (*Eco*RI-B^{H1}) corresponding to the larger of the two fragments produced by *Hae*III digestion of *Eco*RI-B (Table 3).

*Eco*RI generates two fragments, 1.4 kbp (*Eco*RI-F^{H2}) and 3.1 kbp (*Eco*RI-C^{H1}), from *Hae*III-B; similarly sized fragments were generated by *Hae*III cleavage of *Eco*RI-F and *Eco*RI-C, respectively.

The fragment obtained by *Hae*III cleavage of *Eco*RI-C and of 1.9 kbp in size (*Eco*RI-C^{H2}) was derived from the *Hae*III-C fragment, which suggests that *Hae*III-C and -B are neighbors. A 2.45-kbp fragment (*Eco*RI-E^{H1}) is also produced by *Eco*RI from the *Hae*III-C fragment. A cor-

TABLE 3. *Redigestion of the EcoRI fragments from ϕ11 DNA*

*Eco*RI fragment (kbp)	Second digestion	
	*Kpn*I (kbp)	*Hae*III (kbp)
A (9.4)		
B (5.4)		*Eco*RI-B^{H1} (5.25), *Eco*RI-B^{H2} (0.14)
C (5.0)	*Eco*RI-C^{K1} (4.6), *Eco*RI-C^{K2} (0.38)	*Eco*RI-C^{H1} (3.1), *Eco*RI-C^{H2} (1.9)
D (3.7)		
E (3.4)		*Eco*RI-E^{H1} (2.45), *Eco*RI-E^{H2} (0.95)
F (3.25)		*Eco*RI-F^{H1} (1.85), *Eco*RI-F^{H2} (1.4)
G (3.2)		
H (2.75)	*Eco*RI-H^{K1} (1.9), *Eco*RI-H^{K2} (0.85)	
I (1.95)		*Eco*RI-J^{H1} (1.4), *Eco*RI-J^{H2} (0.57)
J (1.7)		
K (1.3)		
L (1.1)		
M (0.245)		
N (0.225)		
O (0.20)		

TABLE 4. *Redigestion of KpnI fragments from phage ϕ11 DNA*

*Kpn*I fragment (kbp)	Second digestion	
	*Eco*RI (kbp)	*Hae*III (kbp)
A (27.1)	*Eco*RI-A (9.4), *Eco*RI-B (5.4), *Eco*RI-C^{K1} (4.6), *Eco*RI-E (3.4), *Eco*RI-H^{K1} (1.9), *Eco*RI-K (1.3), *Eco*RI-L (1.1).	*Hae*III-A^{K1} (19.0), *Hae*III-C (4.35), *Hae*III-B^{K2} (2.7), *Hae*III-E (1.1)
B (15.8)	*Eco*RI-D (3.7), *Eco*RI-F (3.25), *Eco*RI-G (3.2), *Eco*RI-I (1.95), *Eco*RI-J (1.7), *Eco*RI-H^{K2} (0.85), *Eco*RI-C^{K2} (0.375), *Eco*RI-M (0.245), *Eco*RI-N (0.225), *Eco*RI-0 (0.20)	*Hae*III-B^{K1} (11.2), *Hae*III-D (3.2), *Hae*III-A^{K2} (1.4)

TABLE 5. *Redigestion of HaeIII fragments from phage ϕ11 DNA*

*Hae*III fragment (kbp)	Second digestion	
	*Eco*RI (kbp)	*Kpn*I (kpb)
A (20.4)	*Eco*RI-A (9.4), *Eco*RI-B^{H1} (5.25), *Eco*RI-H (2.75), *Eco*RI-K (1.3), *Eco*RI-L (1.1), *Eco*RI-J^{H2} (0.57)	*Hae*III-A^{K1} (19.0), *Hae*III-A^{K2} (1.4)
B (13.8)	*Eco*RI-D (3.7), *Eco*RI-G (3.2), *Eco*RI-C^{H1} (3.1), *Eco*RI-J (1.7), *Eco*RI-F^{H2} (1.4), *Eco*RI-M (0.245), *Eco*RI-N (0.225), *Eco*RI-O (0.20)	*Hae*III-B^{K1} (11.1), *Hae*III-B^{K2} (2.7)
C (4.35)	*Eco*RI-E^{H1} (2.45), *Eco*RI-C^{H2} (1.9)	
D (3.25)	*Eco*RI-F^{H1} (1.85), *Eco*RI-I^{H1} (1.4)	
E (1.1)	*Eco*RI-E^{H2} (0.95), *Eco*RI-B^{H2} (0.14)	

responding 2.45-kbp fragment is found on the *Eco*RI-E fragment by cleavage with *Hae*III. *Hae*III-D is cleaved by *Eco*RI into two fragments. One is 1.85 kbp (*Eco*RI-F^{H1}), which corresponds to the second part of *Eco*RI-F, suggesting that *Hae*III-D and -B are neighbors. Another fragment is 1.4 kbp (*Eco*RI-J^{H1}), which is the same size as the larger of the two fragments obtained by *Hae*III cleavage of *Eco*RI-I, indicating that fragments *Hae*III-D and -A are neighbors. *Hae*III-E, finally, consists of the 0.95-kbp fragment (*Eco*RI-E^{H2}) from the *Hae*III cleavage of the *Eco*RI-E fragment plus the smaller 140-bp fragment (*Eco*RI-B^{H2}) from the *Hae*III cleavage of *Eco*RI-B. These results suggest that *Hae*III-E is located between the *Hae*III-A and -C fragments.

Taken together, digestions of isolated *Hae*III fragments suggest that the restriction fragments are circularly arranged in the order: A-D-B-C-E-A.

End labeling of phage φ11. Our first approach for restriction site mapping of phage φ11 DNA was to use the method described by Smith and Birnstiel (17), which is based on end labeling of the DNA followed by partial digestion to directly map the restriction sites relative to the termini of the DNA molecule. Attempts to end label mature DNA with polynucleotide kinase (10) or by reverse transcription of *Exo*III endonuclease-digested DNA (1) yielded incorporation of radioactivity, but no specifically labeled fragment was found after digestion with restriction enzymes and gel electrophoresis.

Our accumulated results suggest, on the other hand, that there are two *Kpn*I cleavage sites but only two fragments and that the map of the *Hae*III fragments is circular. The failure to obtain discrete bands after end labeling of intact DNA suggests, therefore, that the mature φ11 DNA is circularly permuted.

An alternative method was therefore developed to end label DNA. Blots of partial restriction enzyme digests of DNA were hybridized with a labeled restriction fragment located at the termini of the restricted DNA (J. I. Flock, personal communication). This method was used on isolated *Kpn*I and *Hae*III fragments, for which the end fragments had been identified in the double digests. This approach helped us to determine the relative order of the restriction fragments in the circularly permutated genome.

Partial *Eco*RI digestion of isolated *Hae*III and *Kpn*I fragments. DNA fragments isolated from *Kpn*I and *Hae*III digests were treated with various concentrations of *Eco*RI, and the partial cleavage products were separated on agarose gels. The fragments were transferred to nitrocellulose filters by blotting (18) and hybridized to a nick-translated *Eco*RI-H fragment of phage φ11 DNA which was cloned in pSA2100 in *S. aureus* (8). All partial cleavage products containing fragment *Eco*RI-H^{K1} could be traced after partial cleavage of the *Kpn*I-A fragment with *Eco*RI. The order of the *Eco*RI fragments in *Kpn*I-A could then be determined from the size distribution of the partial fragments. Figure 2A shows the results with partial *Eco*RI digestion of *Kpn*I-A on a 1% agarose gel. The bottom fragment corresponds to the *Eco*RI-H^{K1} frag-

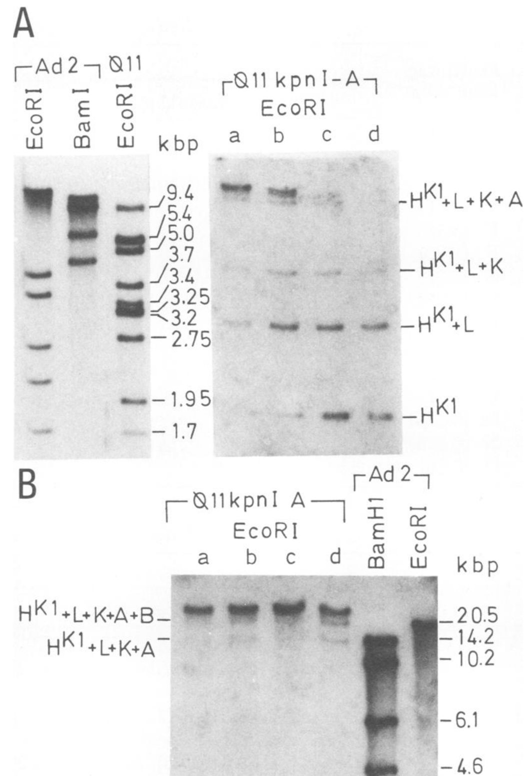


FIG. 2. *Sequential ordering of the EcoRI fragments contained within fragment KpnI-A. Isolated KpnI-A DNA was partially digested with different concentrations of EcoRI. The digested samples were electrophoresed on 0.8% (A) and 0.7% (B) agarose gels with EcoRI fragments of φ11 DNA and BamHI and EcoRI fragments of adenovirus type 2 (Ad2) DNA as size markers. The DNA was then transferred to nitrocellulose filters according to Southern (18). The area of the filters containing the φ11 DNA and Ad2 DNA markers were cut off and hybridized separately to nick-translated φ11 DNA and Ad2 DNA, respectively. The remaining area of the filters were hybridized to the nick-translated EcoRI-H fragment of φ11 DNA, which had been cloned into pSA2100. The filters were analyzed by autoradiography. Lanes a through d indicate increasing concentrations of EcoRI. The partial EcoRI products are indicated on the figure.*

ment (1.9 kbp). The band above corresponds to an addition of *Eco*RI-L, and the third band contains *Eco*RI-H^{K1}, *Eco*RI-L, and *Eco*RI-K. The next band consists of *Eco*RI-H^{K1} + L + K + A, which is better visualized on a 0.7% agarose gel in Fig. 2B. From this gel it is also possible to resolve *Eco*RIH^{K1} + L + K + A + B. Larger fragments are not resolved. From the double digest of ϕ11 DNA it is known that *Eco*RI-C is at the end, which gives the fragment order H^{K1}-L-K-A-B-E-C^{K1} of *Kpn*I-A. The same probe was used for mapping the *Kpn*I-B fragment, whereby the labeled *Eco*RI-H^{K2} fragment (0.85 kbp) from the *Kpn*I/*Eco*RI digest was hybridized to fragments partially digested with *Eco*RI. Figure 3 shows that *Eco*RI-H^{K2} migrates at the bottom of the gel; the band above is *Eco*RI-H^{K1}, which is a contaminant from *Kpn*I-A. *Kpn*I-B was always contaminated with *Kpn*I-A even after two steps of electrophoretic purification. The third fragment from the bottom is *Eco*RI-H^{K2} + I, and the band immediately above is again a contaminant from fragment *Kpn*I-A, *Eco*RI-H^{K1} + L (cf. Fig. 2). The next fragment is *Eco*RI-H^{K2} + I + F or G. The double digest revealed that *Eco*RI-F is in juxtaposition to *Eco*RI-I, and this

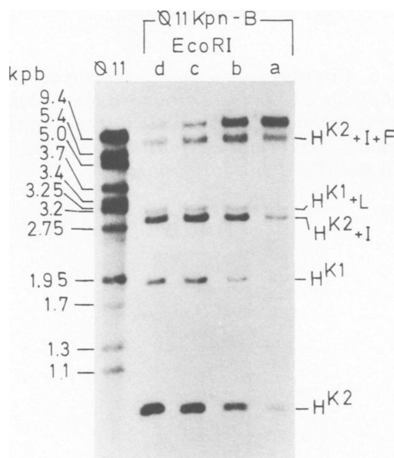


FIG. 3. *Sequential ordering of the EcoRI fragments contained within fragment KpnI-B. Isolated KpnI-B DNA was partially digested with different concentrations of EcoRI. The digested samples were electrophoresed on a 1% agarose gel with EcoRI fragments of ϕ11 DNA as size markers. The DNA was then transferred to nitrocellulose filters according to Southern (18). The area of the filter containing the ϕ11 DNA markers was cut off and hybridized separately to nick-translated ϕ11 DNA. The remaining part of the filter was hybridized to the nick-translated EcoRI-H fragment of ϕ11 DNA, which had been cloned into pSA2100. The filters were analyzed by autoradiography. Lanes a through d indicate increasing concentrations of EcoRI. The partial EcoRI products are indicated on the figure.*

fragment therefore corresponds to *Eco*RI-H^{K2} + I + F. Since the resolution of large fragments is poor with this technique and since the additional *Eco*RI fragments are similar in size (except for the three minor fragments M, N, and O, which are only 200 to 245 bp), fragment *Hae*III-B was also analyzed by partial cleavage with *Eco*RI. The Southern blotting technique was again used, with the *Eco*RI-F cloned in pSA2100 as the labeled probe (8). Figure 4 shows the order of the *Eco*RI fragments in *Hae*III-B: *Eco*RI-F^{H2}-N-O-G-M-D-J-C^{H1}.

From this gel (Fig. 4) it is not possible to accurately determine the size of fragments O, N, and M. Fragment N is slightly larger than fragment O, 270 and 250 bp, respectively, and M is larger than both of them. Although their sizes do not correspond exactly to those observed in 2.2% agarose gels (Fig. 1B), we have tentatively mapped them according to their relative size.

Composite cleavage map. The results of the double digestions and partial cleavages have been summarized in a circular map of phage ϕ11 DNA (Fig. 5). The map order of the *Hae*III fragments yields the first circular restriction enzyme map with the fragment order A-D-B-C-E, where fragments *Hae*III-A and *Hae*III-E are neighbors. The *Kpn*I cleavage sites are then correctly placed on fragments *Hae*III-A and -B. The order of the *Eco*RI fragments has been established by partial cleavage of fragment *Kpn*I-A from *Eco*RI-H^{K1} to *Eco*RI-C^{K1} and on fragment *Kpn*I-B from *Eco*RI-H^{K2} to *Eco*RI-F. Since fragment *Hae*III-B covers the rest of *Kpn*I-B, the remaining *Eco*RI fragments will be obtained from the partial cleavage map of the *Hae*III-B fragment in the order *Eco*RI-F^{H2}-N-O-G-M-D-C^{H1}. Fragment *Eco*RI-C^{H1}, obtained by double digestion of *Eco*RI/*Hae*III, overlaps *Eco*RI-C^{K1}, obtained by double digestion with *Eco*RI/*Kpn*I, which infers that the circular map is complete. These results establish that the linear mature form of phage ϕ11 DNA is circularly permuted.

Restriction enzyme analysis of replicating phage ϕ11 DNA. To compare the restriction of linear and circular forms of ϕ11 DNA, we made an attempt to isolate covalently closed circular DNA from cells infected with ϕ11. Cleared lysates were prepared from strain 8325-4 infected with ϕ11 at 20 min after infection and analyzed by preparative ethidium bromide density centrifugation. The band corresponding to covalently closed circular DNA was collected. This DNA was digested with *Eco*RI and analyzed by agarose electrophoresis. Figure 6 shows a 1% agarose gel with an *Eco*RI pattern identical to that of the mature DNA, again indicating that the mature ϕ11 DNA is circularly permuted.

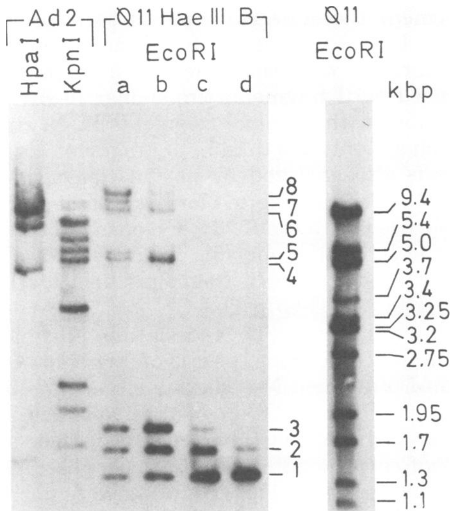


FIG. 4. *Sequential ordering of EcoRI fragments contained within fragment HaeIII-B. Isolated HaeIII-B was partially digested with different concentrations of EcoRI. The digested samples were electrophoresed on a 0.8% agarose gel with EcoRI fragments of φ11 DNA and BamHI, EcoRI, and HpaI fragments of adenovirus type 2 (Ad2) DNA as size markers. The DNA was then transferred to nitrocellulose filters according to Southern (18). The area of the filter containing the φ11 DNA markers was cut off and hybridized separately to nick-translated φ11 DNA. The remaining area of the filter was hybridized to nick-translated Ad2 DNA and the EcoRI-F fragment of φ11 DNA; the latter had been cloned into pSA2100. The filter was analyzed by autoradiography. Lanes a through d indicate increasing concentrations of EcoRI. The partial EcoRI products are indicated by the numbers 1 through 8, where number 8 is fragment EcoRI-F^{H2} + N + O + G + M + D + J + C^{H1}. See text for further details.*

Restriction enzyme map of prophage φ11. Bulk DNA prepared from strain 8325-4(φ11) was digested with *Eco*RI and *Hae*III. The cleavage products were separated on agarose gels. The fragments were then transferred to nitrocellulose filters by the Southern technique (18) and hybridized to nick-translated φ11 DNA. Figure 7 shows the autoradiograms of three gels containing different agarose concentrations. The 0.8% agarose gels reveal that fragment *Eco*RI-B from the chromosomal digest migrates slightly faster than *Eco*RI-B from mature phage DNA. The attachment site of the phage φ11 DNA is therefore probably located on fragment *Eco*RI-B. At integration, fragment *Eco*RI-B is cleaved and the two parts will form the ends of the prophage fused to chromosomal DNA. Two fragments composed of both φ11 and chromosomal sequences will be formed by *Eco*RI digestion. These two fragments could be traced with nick-translated φ11 DNA. One of these migrates close to the *Eco*RI-B fragment of the mature phage φ11 DNA, as shown on the 0.8% agarose gel (Fig. 7). The other is found on the 2.5% agarose gel migrating faster than fragment *Eco*RI-O of the mature phage. This indicates that the attach-

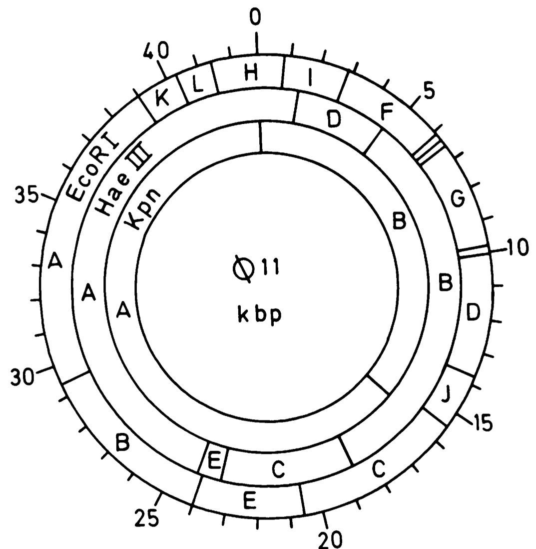


FIG. 5. *Circular restriction endonuclease cleavage map of phage φ11 DNA. The size of the circular map is given in kilobase pairs starting at the KpnI site at 12 o'clock. The relative positions of the EcoRI, HaeIII, and KpnI sites are indicated.*

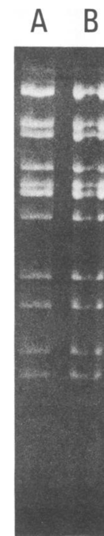


FIG. 6. *Electrophoretic analysis of covalently closed circular (CCC) DNA prepared from strain 8325-4 20 min postinfection with phage φ11. A, Mature φ11 DNA + EcoRI; B, CCC DNA + EcoRI.*

ment site (*att*) of phage ϕ11 DNA is located at one of the ends of the *Eco*RI-B fragment. Since the junction between fragments *Hae*III-A and *Hae*III-E is located 140 bp in from one end of fragment *Eco*RI-B, the location of the *att* site may be revealed from *Hae*III digests of chromosomal DNA. Thus, if *att* is positioned close to the junction between fragments *Eco*RI-B and *Eco*RI-A, *Hae*III cleavage of the chromosomal DNA would create a new fragment with a size of at least 5 kbp containing *Eco*RI-B sequences. No such fragments could be found in a 0.7% agarose gel (Fig. 7B), suggesting that the *att* site is located at the other end of the *Eco*RI-B fragment close to fragment *Eco*RI-E.

Homoduplex analysis of ϕ11 DNA. To confirm that ϕ11 DNA is circularly permuted, homoduplexes of the mature DNA were analyzed in the electron microscope. Figure 8 shows the three different forms of the homoduplexes observed. Figure 8A reveals a homoduplex DNA with single-stranded (ss) tails in both ends. Figure 8B reveals a homoduplex circle with ss tails extending out from two positions on the circle. The distance between the two ss tails corresponds to around 17% of the circumference. Figure 8C shows a duplex circle with doublets of ss tails at two positions on the circle. The distance between the doublets is 31%. The doublets of ss tails are probably created by branch migration, as demonstrated by Lee et al. (6) for coliphage 15. The length of the ss tails was measured on 20 circular molecules and appears to constitute 5.5 ± 2.5% of the length of the mature phage DNA. The distance between the ss tails on the circular homoduplex structure ranged from 0 to at least 42% of the circumference, suggesting that the ϕ11 DNA is randomly permuted.

DISCUSSION

Reciprocal double digestions with restriction enzymes *Eco*RI, *Hae*III, and *Kpn*I of mature and linear DNA from the staphylococcal phage ϕ11 revealed that each *Hae*III fragment is flanked on both sides of another *Hae*III fragment. In addition, two sites were identified for the *Kpn*I enzyme on ϕ11 DNA, but only two fragments were resolved (Fig. 1). A novel ap-

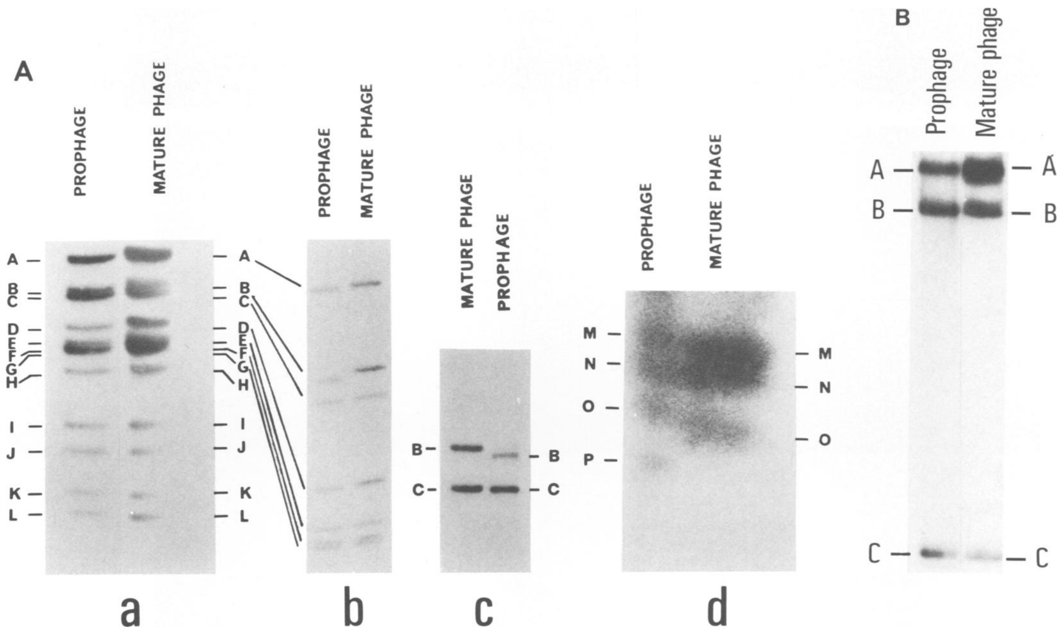
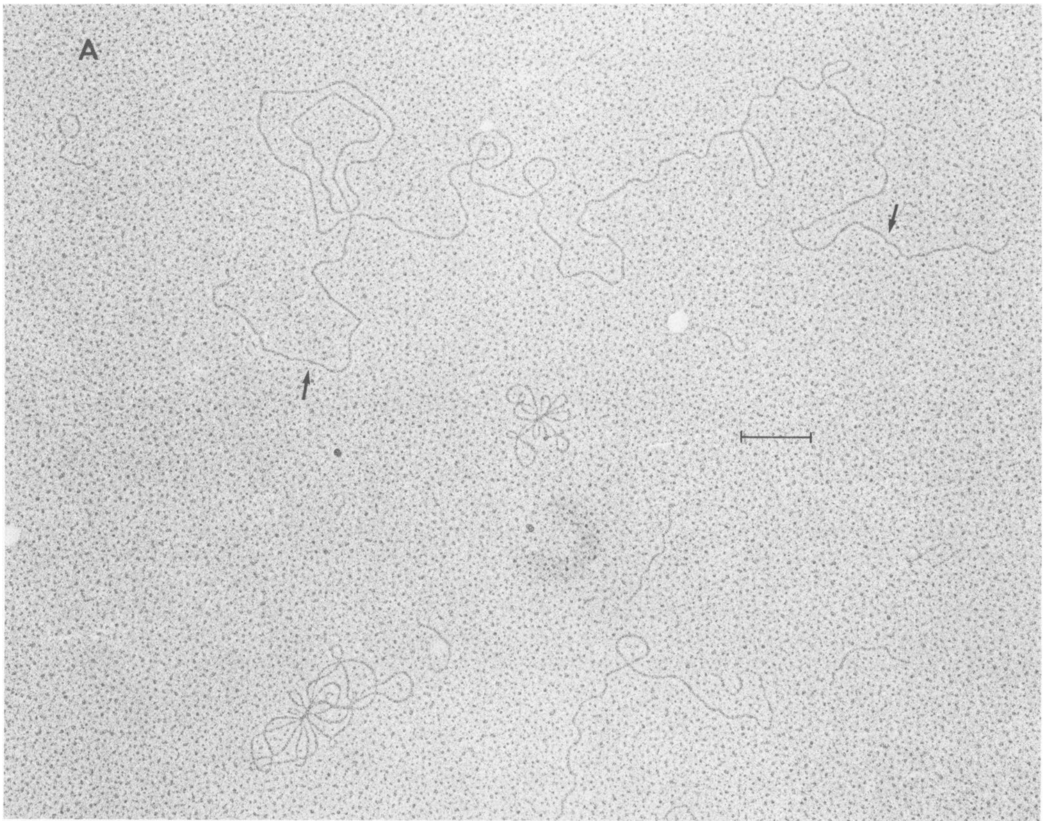


FIG. 7. *Restriction site analysis of prophage ϕ11. (A) Bulk DNA prepared from strain 8325-4 (ϕ11) was digested with restriction enzyme EcoRI and electrophoresed on 1% (a), 0.8% (b, c), and 2.5% (d) agarose gels with EcoRI fragments of mature ϕ11 DNA as a size marker. The DNA was then transferred to nitrocellulose filters according to Southern (18). The filters were hybridized to nick-translated ϕ11 DNA. They were finally analyzed by autoradiography. Note the aberrant migration of the EcoRI-B fragment in gels b and c and the fragment smaller than EcoRI-O in gel d. The gel in c was electrophoresed for an extended period, and only the EcoRI-B and C fragments are shown. (B) The same analysis was performed with HaeIII fragments of chromosomal DNA, where no apparent size differences of the restriction enzyme fragments could be observed in a 0.7% agarose gel. Only fragments A, B, and C are shown on this gel; the remaining fragments, D and E (3.25 and 1.1 kb), have migrated out of the gel.*



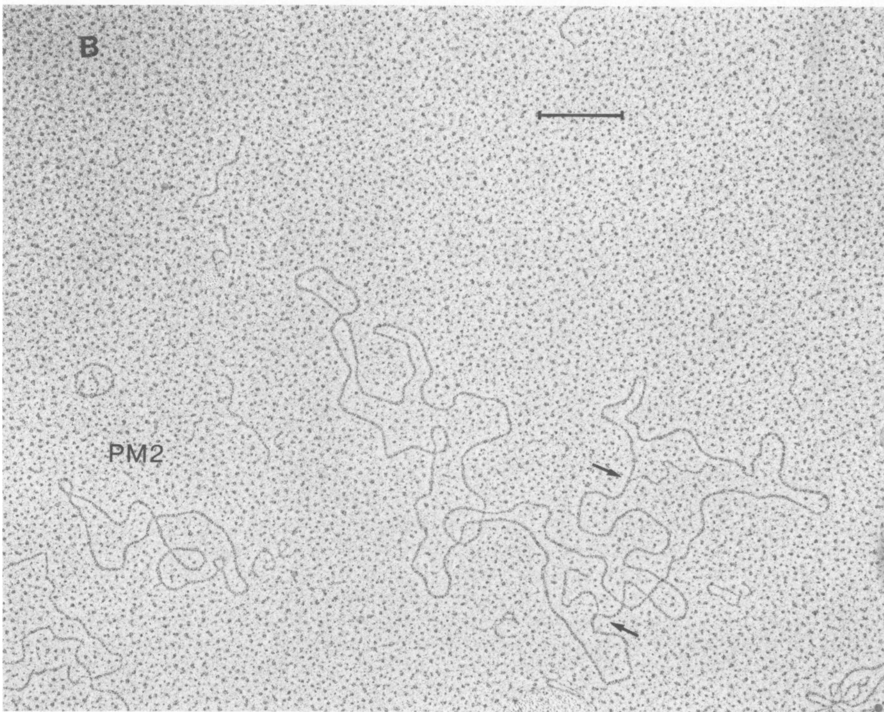


FIG. 8 A AND B

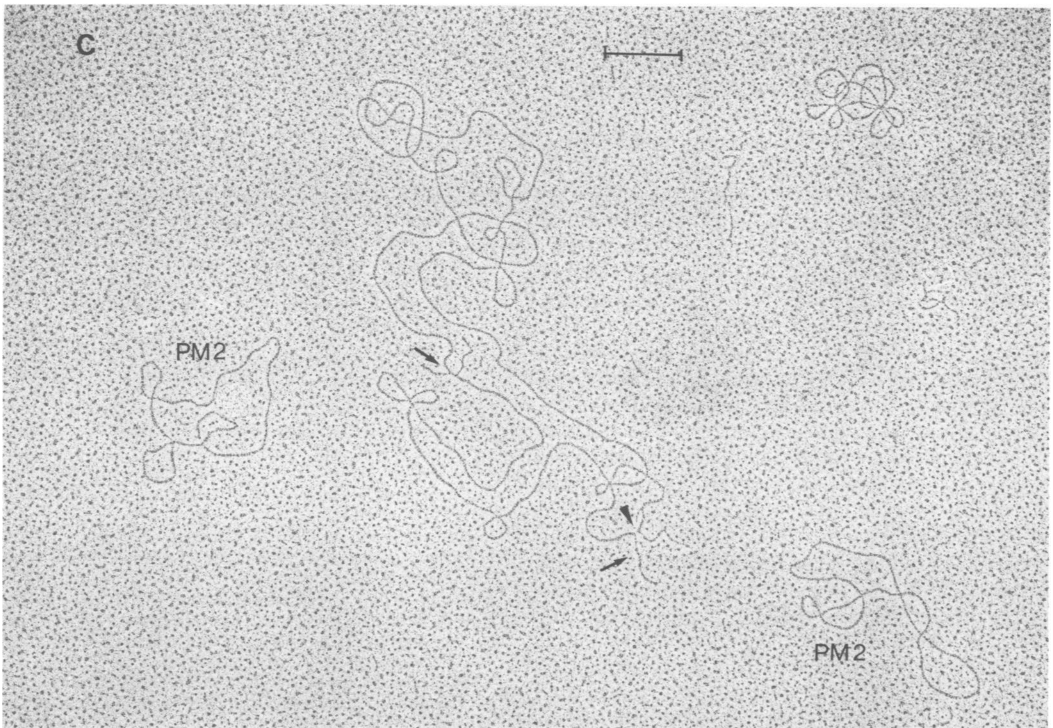


FIG. 8. *Homoduplex analysis of ϕ11 DNA. (A) The duplex linear portion of the molecule consists of 39 kbp. The terminal single-stranded regions are measured to be 9.0 ± 3.5%. The arrows indicate double-strand/single-strand junctions. (B) The duplex circular part of the molecule consists of 43.1 kbp. The terminal repetition is measured to be 4.2%. (C) The duplex circular part of the molecule consists of 43.1 kbp. The terminal repetition is measured to be 5.2%. The arrows point to single-stranded terminal repetitions. Note: This molecule demonstrates single-strand branch migration and branch peel back (▶) in the lower part of the molecule. These spreadings were not restricted with talc. Bar = 1 kbp of duplex DNA.*

proach to the end labeling of partial restriction enzyme digests involving transfer of the DNA to a nitrocellulose filter and probing with a cloned nick-translated end fragment established the relative order of the *Eco*RI restriction enzyme fragments on the mature phage ϕ11 DNA. All of these results together with our failure to trace a restriction fragment by end labeling of intact ϕ11 DNA suggest that the mature ϕ11 DNA has a circular restriction enzyme map, which was established for three restriction enzymes, *Eco*RI, *Kpn*I, and *Hae*III. In addition, the circular early replicative form of ϕ11 DNA appears to contain the same *Eco*RI fragment as the mature phage DNA. These results may all be explained if we postulate that ϕ11 DNA is circularly permuted. This suggestion was confirmed by homoduplex analysis of mature ϕ11 DNA in the electron microscope (Fig. 8), in which double-stranded circles with ss tails were observed. The homoduplex pattern suggests that ϕ11 DNA is permuted to at least 42% of the genome length and that the terminal repetitions correspond to 5% of the genome. Similar structures have been described for homoduplexes of DNA from phage T2 of *Escherichia coli* (20), coliphage 15 (6), and deletion mutants of *Salmonella typhimurium* phage P22 (22), which are all circularly permuted and terminally redundant. Although randomly permuted phage DNA has been observed for phage T2 (9), the randomness of the permutation is probably dependent on the number of genome copies in the concatemers and the size of the terminal repetition whenever there is a unique starting site for encapsidation (23). Tye et al. (23) demonstrated for phage P22 that the wild type containing a terminal repetition of 2% clustered its ends within a region comprising 20% of the genome, but in mutant P22bp1, with a terminal repetition of 7%, the ends were distributed over 70% of the genome. In both cases encapsidation had a unique starting site in the concatemers, and a headful of phage DNA always corresponded to 102% of the size of the circular replicative form of the wild type. Since the terminal repetition in ϕ11 DNA appears to

constitute 5% of the genome, the DNA may still have an unique starting site for encapsidation since with 10 headfuls per concatemer a 50% heterogeneity may be expected in the location of the ends along the genome. Since we cannot determine polarity in the homoduplexes the extent of permutation is unknown, but there must be a minimum of 42% heterogeneity. That no weak additional fragments could be observed in the restriction enzyme digest of ϕ11 DNA (Fig. 1) suggests that ϕ11 DNA is almost randomly permuted, in which case the terminal repetitions will only yield a heterogeneous background after enzyme digestion. From these considerations it can be inferred that the mature phage ϕ11 DNA is longer than the sum of the sizes of the restriction enzyme fragments because half of the terminal repetitions will not be measured with this method. If the terminal repetitions are around 5% of the genome, an overall size of 45 kbp can be calculated for the mature phage DNA, using the total size of the restriction enzyme fragments given in Table 2. This estimate lies within the range determined for the size of the mature ϕ11 DNA by electron microscopy (see Table 1) and as previously reported by Brown et al. (3). Our study has therefore established that the mature ϕ11 DNA is circularly permuted, which correlates well with the circular genetic map established by Kretschmer and Egan (5), with the aid of *sus* mutants of the phage. Correlation of the genetic and physical maps has been accomplished in the accompanying paper (8); several restriction fragments of phage ϕ11 DNA have been cloned in *S. aureus* in order to locate the mutants on specific fragments.

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