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Comparison of the G4 and φX174 Phage Genomes by Electron Microscopy

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The order of G4 *Haemophilus influenzae* Rd (*Hin*dII) DNA fragments was mapped by electron microscopy. G4 *Hin*dII DNA fragments were annealed to φX174 viral single-stranded DNA circles and found to partially hybridize. Eight to eighteen percent of the φX174 DNA circles had a G4 DNA fragment attached, depending upon the fragment and the time of annealing. The G4 *Hin*dII restriction enzyme cleavage map was aligned with the φX174 restriction enzyme cleavage map by analyzing under the electron microscope partial heteroduplex molecules consisting of φX174 single-stranded viral DNA circles annealed with φX174 *Arthrobacter luteus* (*Alu*I) DNA fragments as markers and G4 *Hin*dII DNA fragments. These results indicate that the gene order of the bacteriophages G4 and φX174 is the same. Despite the high frequency of φX174/G4 heteroduplex DNA molecules observed under the electron microscope, heterologous marker rescue experiments with G4 DNA fragments and φX174 mutant viral DNA strand circles were not successful, except in the case of a φX174 amber mutation in gene E (*am*3).

INTRODUCTION

G4 is an isometric φX-like phage isolated from sewage (Godson, 1974). Its single-stranded DNA is approximately 5500 nucleotides long, i.e., approximately the same size as the φX174 DNA which consists of 5375 nucleotides (Sanger *et al.*, 1977). G4 phage codes for the same series of proteins as φX174 but significant differences in electrophoretic mobility of the proteins A, F, G, H, and D of the two phages have been observed (Godson, 1974; Weisbeek *et al.*, 1977) which may reflect differences in size. Analysis of G4–φX174 heteroduplex DNA molecules with the electron microscope shows discrete single-strand loops over 62% of the genome at Tm-31° (Godson, 1974). This may be due to considerable difference in base sequence of the two phages, to size differences between the corresponding genes, to differences in gene order, or to a combination of these factors.

The physical–genetic map of φX174 phage has been well established (Weisbeek *et al.*, 1976; Sanger *et al.*, 1977) and the present study was undertaken to align the G4 and φX174 restriction enzyme cleavage maps and to obtain information on the position and order of the genes of G4 phage on the physical map.

Initially we tried to align the G4 and φX174 maps by heterologous marker rescue. Marker rescue involves annealing of restriction enzyme fragments derived from wild-type RF to mutant single-stranded φX174 DNA followed by spheroplast infection of the partial duplex (DNA (Weisbeek *et al.*, 1976). In the case that the fragment covers the site of the mutation a 100- to 1000-fold increase in the ratio of wild-type over mutant-type progeny phage is observed after spheroplast infection. Using various restriction enzyme fragments the position of a mutation can be determined rather accurately on the physical map of φX174 (Weisbeek *et al.*, 1976). However heterologous marker using *Hin*dII restriction fragments of G4 RF DNA and mutants of single

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stranded φX174 DNA was unsuccessful except for the *am3* mutant in gene E.

Secondly we tried to align the two cleavage maps by electron microscopic analysis of partial heteroduplex molecules obtained by annealing single-stranded φX174 DNA and *Hin*dII restriction fragments of G4 RF DNA. The position of these fragments on the φX174 circle was determined relative to that of *Alu*I φX174 RF fragments with known positions on the φX174 genome (Keegstra *et al.*, 1977). The results align the *Hin*dII restriction map of G4 with the physical map of φX174 and further suggest that the gene order A through F in G4 is the same as in φX174 phage.

MATERIALS AND METHODS

Bacteriophage mutants. φX174 *wt* is the wild-type phage (Sinsheimer, 1959). *am18* (A), *am30* (A), *am86* (A), *am16* (B), *och6* (C), *am42* (D), *am3* (E), and *am9* (G) are conditional lethal mutants and are gifts of Dr. R. L. Sinsheimer (Benbow *et al.*, 1971). Mutants *amH90* (A) and *amN1* (H) are gifts of Dr. M. Hayashi (Hayashi and Hayashi, 1974). *Ts27* (F), *tsH1* (F), and the amber mutant *to8* (B) were obtained from Dr. W. E. Borrias (Borrias *et al.*, 1969).

Bacterial strains. Escherichia coli C (BTCC 122) is the standard sup^- host for φX174. This strain is used to grow and assay *wt* and *ts* phages. *E. coli* HF 4712 (sup^+_{UAG}) is used to grow and assay the *am* and *to* nonsense mutants (Benbow *et al.*, 1974). *E. coli* WWU ($su2_{och}$) is used to grow and plate *och6* (Funk and Sinsheimer, 1970). *E. coli* K58 (sup^+_{UAG} and sup^+_{UAA}) is φX-resistant and is used to make spheroplasts. It is an efficient host for the DNA of all mutants used. Bacteriophage φX174 was grown and plated as described earlier (Weisbeek *et al.*, 1976).

Marker rescue. Fragments were dissolved in 10 m*M* Tris–HCl (pH 8.0), the circular single-stranded DNA in 0.6 *M* NaCl, 0.06 *M* sodium citrate, pH 7.0. Fragments were diluted in 10 m*M* Tris–HCl to $A_{260\ nm} = 0.05$. Ten microliters of this solution were added to 10 μl of circular single-stranded DNA ($A_{260\ nm} = 0.5$), heated for 2 min in a boiling water bath, and incubated for 30 min in a 64° water bath. After cooling the mixture in ice, 0.18 ml of distilled water was added. This solution was used to infect the spheroplast.

Preparation and infection of spheroplasts. Phage DNA was assayed on spheroplasts of *E. coli* K58. Spheroplasts were prepared according to the method described by Guthrie and Sinsheimer (1960), except for the use of PAM medium (Guthrie and Sinsheimer, 1963) instead of nutrient broth. For the assay 0.1 ml of DNA was mixed with 0.1 ml of spheroplasts. After 15 min of adsorption at 30°, 0.8 ml of PAM medium was added and the suspension was shaken for 3 hr at 30°. After adding of 20 μl chloroform the suspension was titrated for infectious phage (average yield 10^8 plaques/ml).

*Restriction enzyme fragments. Hin*dII fragments of G4 RF DNA were separated from a terminal *Hin*dII enzyme digest of G4 RF I DNA by electrophoresis on a 4% polyacrylamide gel and recovered by electroelution (Godson, 1975). The purified DNA fragments were dissolved in 10 m*M* Tris–HCl, pH 8.0. All marker rescue experiments were done with intact G4 *Hin*dII fragments. Most of the marker rescue experiments were also carried out with G4 *Hin*dII fragments which were further digested with a mixture of *Alu*I, *Hae*III, and *Hin*fI to completion in order to decrease the size of these fragments hoping that this would facilitate annealing of homologous regions. However, this made no difference. The restriction enzyme fragments formed by the action of the restriction endonuclease from *Arthrobacter luteus* (*Alu*I fragments) on φX174 RF DNA were a gift of Dr. J. M. Vereijken (Vereijken *et al.*, 1975).

Annealing for electron microscopy. Annealing was carried out in solutions containing single-stranded DNA at a final circle concentration of 20 μg/ml, 0.3 *M* NaCl, 30 m*M* sodium citrate, 10 m*M* Tris–HCl (pH 8.0), and 1 m*M* EDTA. The solution was heated for 2 min at 100° and quenched in ice. Reannealing was carried out for 30 min at 64°. G4 single-stranded DNA and G4 *Hin*dII restriction fragments were mixed at a molar ratio of 1.0.

φX174 single-stranded DNA and G4 *Hin*dII fragments were mixed at a molar ratio of 0.5 and reannealed for 10, 20, and 30 min respectively to determine optimal hybridization conditions for the different fragments.

φX174 single-stranded DNA, φX174 *Alu*I fragments, and G4 *Hin*dII fragments were mixed at a molar ratio of 1:1:1 and reannealed under conditions for optimal hybridization.

Electron microscopy. DNA was spread for electron microscopy by the formamide modifications of the protein monolayer technique (Davis *et al.*, 1971). The spreading conditions used were 50% formamide, 0.1 *M* buffer solution (Keegstra *et al.*, 1977) for the specimens used for mapping and 30% formamide 0.1 *M* buffer solution for the annealing control experiments. φX174 replicative form DNA with at least one nick in one of the strands (RFII) was used as an internal length reference (Lang *et al.*, 1967; Brown and Vinograd, 1974; Danna and Nathans, 1971; Stüber and Bujard, 1977). The specimens were examined and photographed in a Philips EM 301 at a (film) magnification of 26000 × on Kodalith LR film. The magnification was calibrated on each film with a carbon grating replica (Fullam, 2160 lines/mm). Length measurements were carried out with a Hewlett Packard 9864 A digitizer connected to a 9820 A calculator.

G4 and φX174 DNAs. Single-stranded φX174 DNA was isolated from purified φX174 phage (Sinsheimer, 1959a, b) as described earlier (Jansz *et al.*, 1966a). φX174 RF DNA was prepared as described by Jansz *et al.* (1966b). Single-stranded G4 DNA was obtained from purified G4 phage (Godson, 1974) by phenol extraction. G4 RF DNA was prepared as described by Godson and Boyer (1974).

RESULTS

Heterologous Marker Rescue

The results of the heterologous marker rescue experiments with wild-type G4 DNA fragments and mutant φX174 single-stranded DNA are given in Table 1. The ratio of the wild type versus total plaque titer after spheroplast infection was determined for mutant φX174 with (a) and without (b) *Hin*dII G4 RF fragments. The results of Table 1 are expressed as the ratio a/b. In homologous marker rescue of wild-type φX174 fragment DNA with mutant φX174 single-stranded DNA this ratio is approximately 100–1000 if the fragment covers the site of the mutation. In no case was this high ratio observed for the φX174–G4 system indicating that the genetic difference between the two phages is too large to obtain efficient marker rescue for most regions of the genome. However, there may be an exception for the region of the overlapping D/E genes of φX174. The *Hin*dII fragment E of G4 RF consistently gave a ratio of approximately 40 when this fragment was annealed to the φX174 *am3* mutant in gene E (Table 1).

The results were the same whether the *Hin*dII DNA fragment was used intact, or after cutting into smaller pieces with other

TABLE 1

ASSAY OF THE G4 *Hin*dII FRAGMENTS ON MUTANT φX174 SINGLE-STRANDED DNA[a]

	Fragment				
	A	B	C	D/D	E
am18 (A)	—[b]	—	2.1	—	1.2
am30 (A)	—	0.4	0.6	1.5	1.6
am86 (A)	0.7	0.5	—	—	0.6
amH90 (A)	—	—	—	<11	±10[c]
am16 (B)	1.4	0.9	2.2	0.8	±10
to8 (B)	—	—	0.9	—	0.36
och6 (C)	—	0.3	±5	0.7	0.5
am42 (D)	1.7	1.8	1.4	0.6	1.2
am3 (E)	0.7	0.8	0.6	1.7	40
ts27 (F)	0.4	2	2.2	—	0.62
tsH1 (F)	<5	0.13	2	0.2	0.3
am9 (G)	1.7	0.1	1.7	0.55	0.1
amN1 (H)	2.5	1	0.04	1.3	0.05

[a] The fragment assay is described under Materials and Methods. The ratio of wild type to total phage was determined with (a) and without (b) the fragment. The figures represent the ratio a/b.

[b] Not determined.

[c] Approximately.

restriction endonucleases. This was done in order to reduce interference of nonhomologous regions with homologous (or more homologous) regions.

HindII Restriction Fragment Map of G4

For mapping the G4 *Hin*dII restriction enzyme on G4 single stranded DNA, we used the *Hin*dII fragments A and C as markers. The partial duplexes formed are composed of G4 single stranded DNA, G4 *Hin*dII restriction fragments A and C, and the fragment to be mapped (B, D, or E). The G4 *Hin*dII restriction endonuclease fragments A, B, C, and E were single fragments, whereas the fragment D consists of two species (Godson, 1975). In every set of hybrids ϕX174 RF II DNA was added as an internal length standard. The mean values of the lengths of the fragments and the spacing between them in the hybrid series are listed in Table 2. The internal length reference (ϕX174 RF II) was used to correct for single-strand stretch assuming that the length of ϕX174 and G4 RF II is the same. The length of the ϕX174 RF II in each specimen was also used to scale the data in the experiments, so they could be compared (Keegstra *et al.*, 1977). With the three partial duplexes used (Fig. 1) we can construct the G4 *Hin*dII map (Fig. 2), which map is the same as found earlier from analysis of partial restriction digests (Godson, 1975).

Alignment of the G4 and ϕX174 Restriction Enzyme Cleavage Maps

Reannealing in periods of 10, 20, and 30 min were used to find the optimum hybridization conditions for the different G4 fragments on the ϕX174 single-stranded circle. For each specimen the number of circles with a fragment and without a fragment were determined (Table 3). The 30-min hybridization period was optimal for the smaller fragments (D and E). The larger fragments needed a short reannealing period. The decrease of the number of circles with a fragment by increasing the reannealing period is probably due to the greater stability of the homoduplex (G4 DNA fragments) compared to the heteroduplex (ϕX174 DNA–G4 DNA fragments).

The ϕX174 *Alu*I marker fragments which were used to map the G4 *Hin*dII fragments on ϕX174 single-stranded circles are listed in Table 4. Attempts to map the G4 *Hin*dII fragment A were not successful. The struc-

TABLE 2

LENGTH DATA FOUND IN THE DIFFERENT G4 PARTIAL DUPLEX SERIES[a]

Hybrid	RF II ϕX174 (μm)	Length of ds parts	Length of ss parts
ss-A–B–C	1.91 ± 0.07 (16)	A:0.90 ± 0.04 (26) B:0.34 ± 0.03 (22) C:0.25 ± 0.03 (27)	A–C: 0.20 ± 0.03 (8) C–B: 0.32 ± 0.03 (6)
ss-A–C–D	1.86 ± 0.08 (16)	A:0.86 ± 0.03 (34) C:0.23 ± 0.02 (29) D:0.18 ± 0.02 (41)	C–D: 0.13 ± 0.02 (12) D–A: 0.34 ± 0.02 (28)
ss-A–C–E	1.83 ± 0.04 (14)	A:0.83 ± 0.04 (60) C:0.24 ± 0.02 (35) E:0.10 ± 0.01 (42)	A–C: 0.20 ± 0.02 (34) E–A: 0.50 ± 0.04 (29)

[a] The molecules were spread by the formamide modification of the protein monolayer technique. RF II was an internal length standard for double-stranded (ds) ϕX174 DNA in each series. All the single-stranded (ss)-lengths were corrected for stretch during mounting for electron microscopy. The figure between brackets is the number of molecules used.

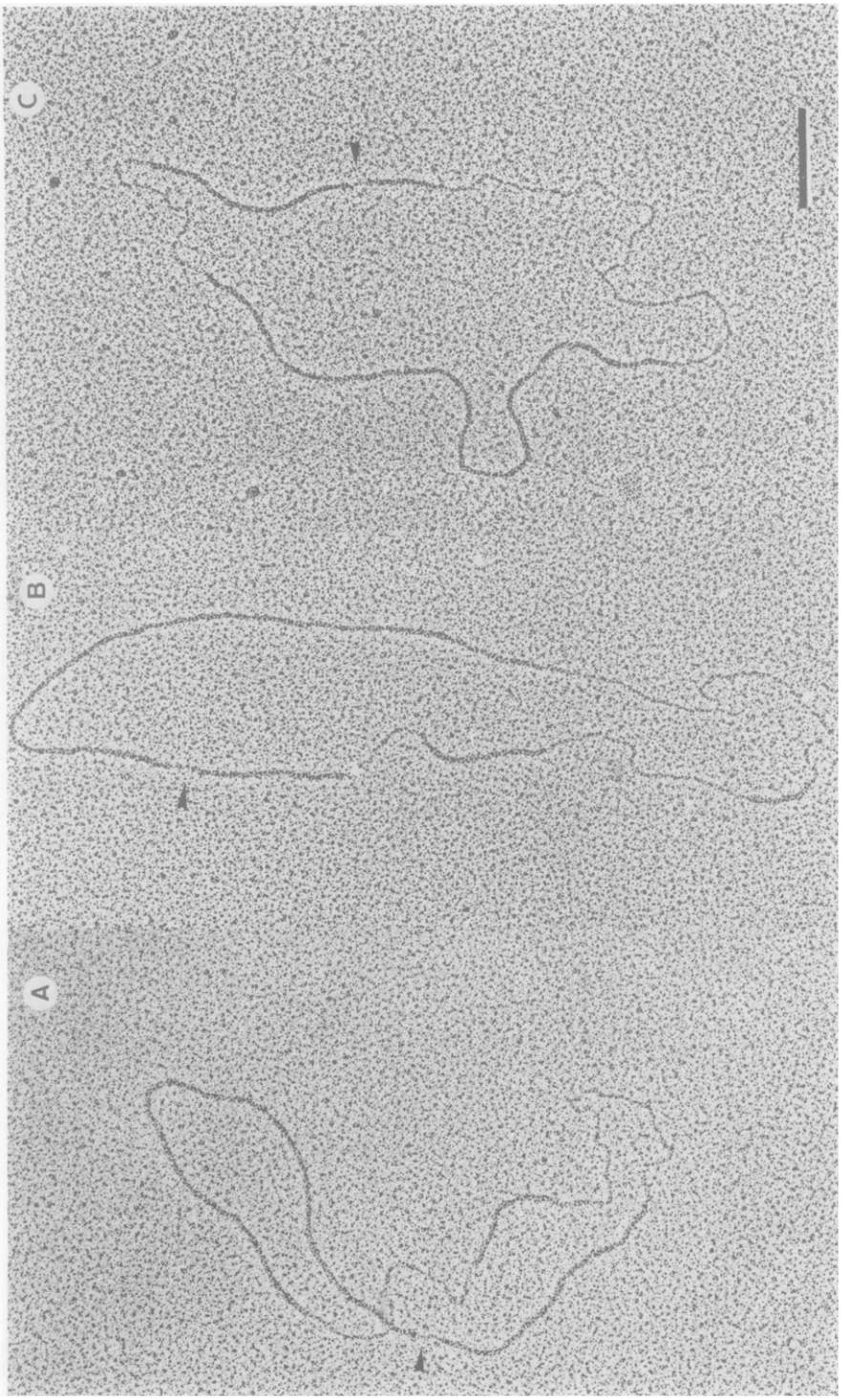

FIG. 1. Electron micrographs of the partial duplex molecules of G4 DNA and *Hin*dII G4 fragments. Hybrids with the fragments A, B, and C (a); A, C, and D (b); and A, C, and E (c). The scale line represents 0.1 μm. The arrow is pointing at a small gap that was present in some of the hybrids. These gaps are probably the result of some exonuclease activity.

FIG. 2. A diagram of the three partial duplexes (Fig. 1) normalized to unit length. At the top of this figure is shown the G4 *Hin*dII map constructed from the lower partial duplex series.

tures of G4 *Hin*dII fragment A and ϕX174 single-stranded circles were too complex to be analyzed. Strand separation of the A fragment may overcome this difficulty. The partial duplexes used to map the other G4 *Hin*dII fragments relative to the ϕX174 *Alu*I map (Fig. 3) gave the information required for the alignment of the two maps (Table 4). Because of the partial homology between ϕX174 and G4, the part of the G4 fragments that becomes hybridized onto the ϕX174 single-stranded circle varies considerably. However, most of the G4 *Hin*dII fragments hybridized well on ϕX174 single-stranded DNA (at least) in the middle of the fragment. To map the G4 fragments the mean values were used and the center of the region at which the G4 fragment is attached was determined relative to the marker fragments. These data were used to align the G4 *Hin*dII map with the ϕX174

TABLE 3

REANNEALING PERIODS FOR THE HETEROLOGOUS ϕX174–G4 HYBRIDIZATIONS[a]

G4 *Hin*dII fragment	Reannealing period (min)		
	10	20	30
A	18.5[b]	13.6	9.5
B	8.3	14.1	8.4
C	9.4	16.1	10.1
D	13.3	15.0	16.1
E	10.0	13.8	16.3

[a] The reannealed samples were spread for electron microscopy by the formamide modification of the protein monolayer technique. For each specimen about 500 molecules were inspected. The numbers in the table are the amount of circles with a fragment, divided by the total number of molecules observed and multiplied by 100%.

[b] Results are in percentages.

TABLE 4

SUMMARY OF LENGTH DATA FOUND IN THE DIFFERENT HETEROLOGOUS ϕX174–G4 HYBRID SERIES[a]

Hybrid ϕX174–G4	RF length ϕX174 (μm)	Length of ds parts	Length of ss parts
ss-A3–A4–B	1.93 ± 0.07 (54)	A3: 0.23 ± 0.03 (84)	A3–A4: 0.44 ± 0.05 (70)
		A4: 0.12 ± 0.02 (72)	A4–B: 0.70 ± 0.09 (33)
		B: 0.18 ± 0.06 (48)	B–A3: 0.18 ± 0.05 (53)
ss-A2–A8–C	2.09 ± 0.07 (50)	A2: 0.27 ± 0.03 (86)	A2–A8: 0.42 ± 0.05 (61)
		A8: 0.08 ± 0.02 (62)	A8–C: 0.40 ± 0.08 (45)
		C: 0.15 ± 0.07 (70)	C–A2: 0.53 ± 0.09 (69)
ss-A2–A8–D	1.93 ± 0.08 (72)	A2: 0.27 ± 0.03 (95)	A2–A8: 0.41 ± 0.06 (68)
		A8: 0.09 ± 0.03 (69)	A8–D1: 0.30 ± 0.06 (30)
		D1: 0.15 ± 0.04 (49)	D1–D2: 0.32 ± 0.08 (5)
		D2: 0.12 ± 0.06 (20)	D2–A2: 0.22 ± 0.07 (20)
ss-A3–A4–E	2.00 ± 0.07 (44)	A3: 0.22 ± 0.02 (55)	A3–A4: 0.44 ± 0.06 (46)
		A4: 0.12 ± 0.02 (46)	A4–E: 0.36 ± 0.07 (19)
		E: 0.08 ± 0.02 (20)	E–A3: 0.67 ± 0.05 (20)

[a] The specimens were mounted for electron microscopy by the formamide modification of the protein monolayer technique. The hybrids were corrected for single-strand stretch using the value of the double-stranded length standard on the same area of the specimen. Scaling of the data was done using the mean value of the internal length standard. For further details see Results.

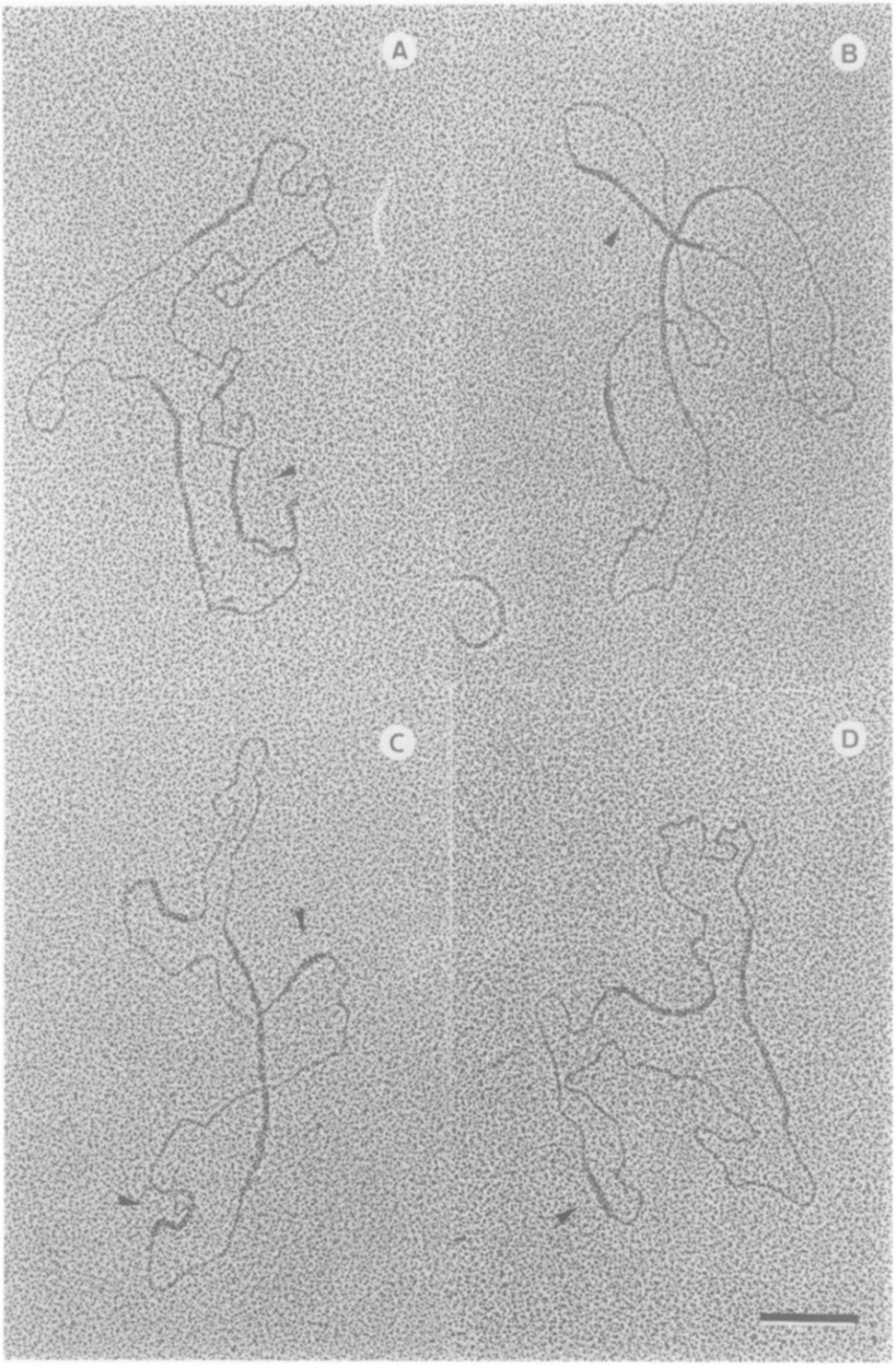

FIG. 3. Electron micrographs of partial duplex molecules of ϕX174 DNA and *Hin*dII G4 fragments and *Alu*I ϕX174 fragments. Partial duplexes with the fragments A3, A4, and B (a); A2, A8, and C (b); A2, A8, and D (c); A3, A4, and E (d). The arrow shows the G4 fragment. The loops shown in the figure are not specific but represent unpaired regions present in some of the molecules. The scale line is 0.1 μm.

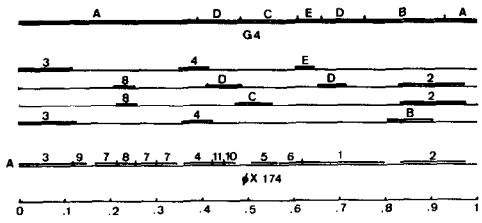

FIG. 4. A diagram of the four partial duplexes (Fig. 3) normalized to unit length. At the bottom of this figure is shown the *Alu*I map as found by electron microscopy (Keegstra *et al.*, 1977) and at the top the aligned G4 *Hin*dII map.

*Alu*I map (Fig. 4). Unpaired regions were observed in all partial heteroduplexes, however, they occur only in a small fraction of the molecules (about 10%) and in different regions of the molecules and therefore could not be mapped accurately. In the partial duplexes in which the ϕX174 *Alu*I fragments A2 and A8 were used as markers (i.e., ϕX174 A2, A8, G4 *Hin*dII C and ϕX174 A2, A8, G4 *Hin*dII D) additional small fragments (contaminating fragments) were found (about 10%) at the position of A10–A11 on the ϕX174 *Alu*I map. These contaminating fragments represent the A10–A11 partial digestion product which is present in preparations of A8 (Keegstra *et al.*, 1977).

The single-stranded lengths in the partial duplexes were corrected with the value of the internal length standard (RF II) in the same area in the specimen as the hybrid. The lengths of the duplex regions in each partial heteroduplex add up to d and the single-stranded regions were corrected so that their total length is RF II length $- d$. The corrected hybrids were then scaled on the mean length of the internal length standard. To align the two maps the mean value of the partial duplexes were normalized to unit length.

DISCUSSION

This study was undertaken to align the restriction enzyme cleavage maps of bacteriophages ϕX174 and G4 and to obtain, from this alignment, information on the genetic map of G4. The number and position of endonuclease restriction cleavage sites in G4 and ϕX174 are quite different (Godson, 1975; Hayashi and Hayashi, 1974; Weisbeek *et al.*, 1976). This has led to confusion about the proper alignment of the G4 and ϕX174 restriction enzyme cleavage maps.

Although G4 and ϕX174 DNA differ considerably in base sequence (Godson, 1974; Shaw *et al.*, 1978), there is enough sequence homology for hybridization. G4 *Hin*dII restriction fragments B, C, D, and E hybridize reasonably well to the ϕX174 single-stranded circle and their order could be determined (Fig. 4). The position of the G4 *Hin*dII fragment A, which could not be determined directly is inferred from the open space on the ϕX174-G4 hybrid map (Fig. 4).

The place where the G4 fragment is attached to the ϕX174 single-stranded DNA is unique. None of the fragments is found at more than one position, except for the G4 *Hin*dII fragment D, which is also found twice on the G4 single-stranded DNA. The position of the G4 *Hin*dII fragments on the ϕX174 circle was determined relative to that of *Alu*I fragments of ϕX RF (Fig. 4). This procedure aligned the physical map of G4 with the physical–genetic map of ϕX174 (Fig. 5). The results show that the order of the G4 *Hin*dII fragments on the ϕX174 single-stranded DNA is the same as the order on the G4 single-stranded DNA (Figs. 2 and 4). The consecutive G4 fragments D, C, E, D, and B hybridize to regions of the ϕX174 circle which correspond to the genes A/B, C, D/E, J, and F respectively. This indicates that the genes A through F in G4 phage occur in the same order as in ϕX174.

No conclusion can be drawn about the order of the genes G and H in G4 phage. Under the electron microscope 8–18% of the ϕX174 circles have a G4 fragment attached. Nevertheless no rescue of the G4 wild-type allele has been observed, with exception of the ϕX174 gene E. Possible explanations for this discrepancy between hybridization and rescue are (1) the sequence homology between the G4 DNA fragment and the ϕX ring is too low for a proper incorporation of the G4 fragment in the newly synthesized complementary strand;

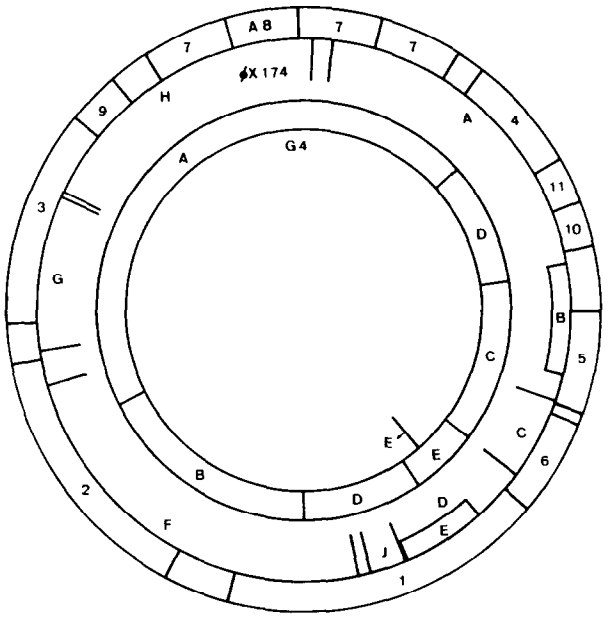

FIG. 5. Combination of the maps of Fig. 4 and the genetic map of φX174 (Weisbeek *et al.*, 1976; Sanger *et al.*, 1977) and the start of gene E of G4. The outer fragment map represents the φX174 *Alu*I map, the next map is the genetic map of φX174. The inner map is the aligned G4 *Hin*dII map. The G4–gene E start in the G4–E fragment which position is known from sequence data (Godson, unpublished) is indicated.

(2) the base differences between fragment and ring induce a repair process that results in either complete removal of the fragment or the formation of a completely complementary DNA fragment; (3) the effective incorporation of the fragment in the new complementary strand leads to the formation of a hybrid φX174–G4 strand. When the genetic information of the G4 part does not fit in with the genetic content on the φX174 part of this DNA molecule, then a nonviable hybrid is formed and no wild-type progeny phage will be found. Although as yet no rigid discrimination between these possibilities can be made, the amount of base pairing of the fragment with the DNA ring (especially at the termini of the fragment) will have a large effect on its chance of being incorporated in the new complementary strand.

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