Arthrobacter Luteus Restriction Endonuclease Cleavage Map of ϕ X174 RF DNA

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Cleavage of ϕ X174 RF DNA with the restriction endonuclease from *Arthrobacter luteus* (*Alu* I) produces 23 fragments of approximately 24-1100 base pairs in length. The order of most of these fragments has been established by digestion of *Haemophilus influenzae* Rd (*Hind* II) and *Haemophilus aegyptius* (*Hae* III) endonuclease fragments of ϕ X RF with *Alu* I and by reciprocal digestions of *Alu* I fragments with *Hind* II and *Hae* III. In this way the *Arthrobacter luteus* map could be aligned with the *Hind* II and *Hae* III cleavage maps of ϕ X174 RF DNA of A. S. Lee and R. L. Sinsheimer ((1974) *Proc. Nat. Acad. Sci. USA* 71, 2882-2886).

INTRODUCTION

The double-stranded form (RF¹) of the genome of bacteriophage ϕ X174 has been cleaved by the restriction endonucleases of *Haemophilus influenzae* Rd (*Hind* II enzyme²) into 13 fragments (Edgell *et al.*, 1972; Lee and Sinsheimer, 1974a), *Haemophilus aegyptius* (*Hae* III enzyme³) into 11 fragments (Middleton *et al.*, 1972; Lee and Sinsheimer, 1974a), *Haemophilus parainfluenzae* into 8 fragments (Johnson *et al.*, 1973; Lee and Sinsheimer, 1974a), *Haemophilus aphrophilus* into 5 fragments and *Haemophilus influenzae* H-I

into 8 fragments (Hayashi and Hayashi, 1974). The order of most of these fragments has been established and the approximate location of a number of the restriction endonuclease cleavage sites on the genetic map of ϕ X (Benbow *et al.*, 1971) have been placed (Chen *et al.*, 1973; Hayashi and Hayashi, 1974; Lee and Sinsheimer, 1974b).

These cleavage maps have already made possible an analysis of a number of interesting functions of ϕ X-DNA. Lee and Sinsheimer (1974b) have identified the position of the only methylcytosine in ϕ X-DNA in the *Hind* II fragment R6C and the overlapping *Hae* III fragment Z2. The *Hind* II enzyme fragments R2, R4 and R6 and the overlapping *Hae* III enzyme fragments Z1, Z2 and Z3 have been shown to contain binding sites for RNA polymerase which presumably represent three separate promoter regions of the ϕ X genome (Chen *et al.*, 1973). RF replication, studied by terminus labeling, has been shown to start in the R3 fragment position (within cistron A) and to proceed clockwise around the genetic map (cistrons A-H) in one direction only (Godson, 1974) in confirmation of genetic evidence on the localization of the

¹ Abbreviations used: ϕ X RF, double-stranded circular replicative form DNA of ϕ X174; ϕ X RF I, supercoiled ϕ X RF with both strands closed; ϕ X RF II, nicked RF I; *Hind* II and *Hind* III, *Hae* II and *Hae* III, and *Alu* I are restriction endonucleases from *H. influenzae* Rd, *H. aegyptius*, and *Arthrobacter luteus*, respectively. R, Z and A refer to DNA fragments produced by *Hind* II, *Hae* III and *Alu* I, respectively. These fragments have been numbered according to their size as determined by gel electrophoresis, starting with the largest, e.g., R1, R2, R3, etc. TCA, trichloroacetic acid; b.p., base pairs.

² *Hind* III does not cleave ϕ X RF (Brown and Vinograd, 1974).

³ This fragmentation pattern is due to *Hae* III and not *Hae* II (Roberts *et al.*, 1975).

origin and direction of ϕ X RF replication (Baas and Jansz, 1972). Similarly, the origin of ϕ X174 single-stranded DNA synthesis, late in infection, has been localized at the position of the *Hind* II R3 fragment (Johnson and Sinsheimer, 1974; Godson, 1974).

For exact localization of these and other interesting functions on the ϕ X genome and complete nucleotide sequence analysis the cleavage of ϕ X RF into small fragments, 50–100 nucleotides in length, is desirable which requires additional restriction endonucleases with unique cleavage specificity.

Restriction endonucleases isolated from a number of bacterial strains have been tested on ϕ X RF by Dr. R. Roberts (personal communication). For the isolation of small fragments the *Alu* I digestion pattern showed great promise. In the present investigation ϕ X RF has been cleaved with *Arthrobacter luteus* restriction endonuclease into 23 fragments. Most of these fragments have been ordered and aligned with the *Hind* II and *Hae* III cleavage maps of ϕ X RF by using the following approach. By partial and complete digestions of *Hind* II fragments with *Alu* I enzyme followed by length analysis of the resultant products, the position of a number of *Alu* I fragments and the linkage of some of the *Hind*-*Alu* subfragments within *Hind* II fragments could be established. In a similar approach the *Hae* III fragments were analysed with *Alu* I. Also, reciprocal digestions of *Alu* I fragments were carried out. The combination of these data yielded a partial *Alu* I cleavage map in which the position of 18 of the *Alu* I fragments could be unequivocally established.

MATERIALS AND METHODS

Isolation of uniformly labeled (32 P) ϕ X RF I. Bacteriophage ϕ X174 and the host *Escherichia coli* strain C were obtained from Dr. R. L. Sinsheimer. The growth medium contained per liter: 1 g of NH_4Cl , 2 g of NaCl , 0.5 g of KCl , 6 g of Tris , 15 g of dephosphorylated casamino acids, 30 g of glycerol, 0.61 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 44 mg of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 1 ml of 1% gelatin solu-

tion. The pH was adjusted with HCl to 7.0. Carrier-free $\text{H}_3 \text{ }^{32}\text{PO}_4$ (The Radiochemical Centre, Ltd., Amersham), 20 $\mu\text{Ci/ml}$, was added 5 min after infection. ϕ X RF was prepared as described previously (Jansz *et al.*, 1966). Pure RF I was isolated by CsCl buoyant density centrifugation in the presence of 200 $\mu\text{g/ml}$ of ethidium bromide (Radloff *et al.*, 1967). The average specific activity of RF I was 1×10^6 cpm/ μg of DNA.

Preparation of restriction endonucleases. *Haemophilus influenzae* Rd was obtained from Dr. W. Fiers, *Haemophilus aegyptius* from Dr. J. G. G. Schoenmakers, and *Arthrobacter luteus* from Dr. R. Roberts.

H. influenzae Rd and *H. aegyptius* were grown in brain-heart infusion medium (Difco) containing hemin (Eastman; 10 $\mu\text{g/ml}$) and NAD (Sigma; 2 $\mu\text{g/ml}$) to an OD_{650} of 1.0. The cells were harvested by centrifugation and the enzymes were prepared essentially as described previously (Takanami and Kojo, 1973; Takanami, 1973). After the phosphocellulose column aliquots of the fractions were assayed for restriction endonuclease activity by resolving the hydrolysates of ϕ X RF I DNA on polyacrylamide-gel electrophoresis. Separately, aspecific nuclease activity was assayed by incubation with ϕ X ^{32}P -labeled RF II and measuring TCA-soluble counts. Fractions which yielded a characteristic pattern of fragments and which showed little aspecific nuclease activity were, after dialysis, rechromatographed on phosphocellulose. After this second phosphocellulose column no TCA-soluble counts could be detected in most of the fractions which had restriction endonuclease activity. These fractions were concentrated by dialysis against 50% glycerol and stored at -20° . In this way a mixture of *Hind* II and *Hind* III was prepared from *H. influenzae* Rd and *Hae* III from *H. aegyptius*. (No *Hae* II activity was detected in the phosphocellulose fractions).

Arthrobacter luteus was grown in nutrient broth (Guthrie and Sinsheimer, 1960) to an OD_{650} of 1.0. The preparation of the restriction endonuclease was the same as for the *Haemophilus* enzymes, except that

the second phosphocellulose column was not required.

Preparation and analysis of endonuclease cleavage fragments. Digestions of ^{32}P -labeled RF I with restriction endonucleases were carried out in 50 mM NaCl, 6.6 mM Tris (pH 7.5) and 6.6 mM MgCl_2 for 3 hr at 37°. The required amount of restriction endonuclease was determined in pilot experiments. The reactions were stopped by adding EDTA to 10 mM and SDS to 1%. After incubation for 15 min at 37° sucrose and bromophenol blue tracking dye were added to 20 and 0.5%, respectively. The digests were then subjected to electrophoresis for 16–20 hr at 140–170 V on 2-mm-thick $\times$ 18-cm-wide $\times$ 40-cm-long slab-gels of varying polyacrylamide concentrations in 0.04 M Tris, 0.02 M sodium acetate and 2.0 mM EDTA, pH 7.8 (Loening, 1968). The gel chamber used was similar to that described in the literature (Reid and Bielecki, 1968; De Wachter and Fiers, 1971). Autoradiography was performed with Kodak Royal X-omat medical X-ray film on the wet gels covered with Saran wrap. ^{32}P -DNA fragments were recovered from gels by excision with a scalpel followed by electrophoresis. The ^{32}P -DNA fragments were precipitated by adding two volumes of 100% ethanol, 0.1 volume of 3 M sodium acetate (pH 5.5) and cooling overnight at –20°. After centrifugation for 30 min at 22,000 rpm the fragments were taken up in small volumes of digestion buffer. The ^{32}P -DNA fragments were redigested with appropriate amounts of other restriction enzymes under the same incubation conditions used for the digestion of intact ^{32}P -labeled RF I. The secondary fragment digests were subjected to electrophoresis on polyacrylamide slab-gels, as described above, together with the undigested fragment as a control for purity. On the slab gels digests of ^{32}P -labeled RF I, with the restriction enzymes in question, were also run. These digests were used for calibration of the gels.

The radioactivity of the fragments was determined by measuring the Cerenkov radiation of excised gel fragments in a scintillation counter.

RESULTS

*Number and Molecular Size of *Alu* I Fragments of ϕ X RF*

Cleavage of ^{32}P -labeled RF with *Alu* I endonuclease yielded 13 radioactive bands on 3% polyacrylamide gels (Fig. 1A). In addition the ϕ X RF cleavage products were analysed on 5% polyacrylamide gels and on a combination gel of 3% and 10%. (Figs. 1B and C). The results indicate that ϕ X RF is cleaved by *Alu* I into at least 17 fragments. By comigration in the same gels the fragmentation pattern of ϕ X RF by *Hind* II is shown.

In order to determine the molecular size (number of base pairs) of the *Alu* I fragments, the mobilities of the *Alu* I bands were compared with those of the *Hind* II fragments, the sizes of which have been well established (Lee and Sinsheimer, 1974). The migration of the *Hind* II bands on the 3% gel was linearly related to the log of their nucleotide length (Fig. 2A); from this plot the length of the *Alu* I bands A1–A12 was estimated. Similar results were obtained for the *Alu* I bands on 5% gels (Fig. 2B). By extrapolation of the straight line the size of the small bands A13–A16 could also be estimated. The length of band A17 was determined on a 7.5% polyacrylamide slab-gel. From the relative yield of each band from uniformly labeled ^{32}P - ϕ X RF it was concluded that band A7 is threefold, A12 is fourfold and A15 is twofold (Fig. 3).

Table 1 summarizes the molecular size estimates as well as the fraction of the genome of each *Alu* I fragment.

*Order of *Alu* I Fragments by Digestion of *Hind* II Fragments with *Alu* I Endonuclease*

The general approach for ordering the *Alu* I fragments was the partial and complete digestion of ^{32}P -labeled *Hind* II fragments with *Alu* I followed by size estimates of the resultant products on 3% polyacrylamide slab-gels. Combination of these data yielded the linkage of *Hind*-*Alu* subfragments within each of the *Hind* II fragments.

FIG. 1. Autoradiograms of fragmentation patterns of ^{32}P -labeled ϕX RF DNA, digested with the restriction endonucleases from *H. influenzae* Rd and from *Arthrobacter luteus*. The *Hind* II and *Alu* I digests comigrated in polyacrylamide slab-gels of 3% (A) and 5% (B) and in a combination gel in which the upper part was 3% polyacrylamide and the lower part 10% (C).

The results are summarized in Table 2. The data indicate that the *Alu* I fragment A 4 is situated within *Hind* II fragment R3, A7 in R4, another A7 in R6, A9 in R2, A10 in R8, A12 in R5, another A12 in R1, A13 in R1, A14 in R4 and A15 and A16 (or A17) in R5. These data and the linkage data from Table 2 in several alternative combinations are shown in Fig. 4A, which represents the *Hind* II map of ϕX RF (Lee and Sinsheimer, 1974a).

Order of *Alu* I Fragments by Redigestion of *Alu* I Fragments with *Hind* II Enzyme

The next step was the determination of

the linkage between *Hind*-*Alu* subfragments at the *Hind* II cleavage sites in ϕX RF. The approach was the digestion of *Alu* I fragments with *Hind* II enzyme in order to identify those *Alu* I fragments which overlap the *Hind* II cleavage sites.

^{32}P -labeled *Alu* I fragments, A1 to A11 were redigested with *Hind* II followed by size estimates of the digestion products on 3% polyacrylamide slab-gels. The results are shown in Table 3.

From the data in Table 3 in combination with the linkage data as presented in Fig. 4 and Table 2, part of the *Alu* I map of ϕX RF could be deduced as follows.

FIG. 2. Length analysis of the DNA bands produced by complete digestion of ^{32}P -labeled ϕ X RF with the *Arthrobacter luteus* restriction enzyme. *Alu* I and *Hind* II digests of ^{32}P -labeled ϕ X RF were coelectrophoresed in a 3% polyacrylamide slab-gel (A; see Fig. 1A) and in a 5% polyacrylamide slab gel (B; see Fig. 1B). The migration distances of the *Hind* II fragments were plotted against the logarithms of their lengths in base pairs; this resulted in a straight line. The number of base pairs of each *Alu* I band was then determined by matching its migration distance onto the straight line, as indicated by the arrows.

A7 is the only overlapping *Alu* I fragment that fits the R4-R3 junction. The combination (70 + 190) base pairs corresponds in length and *Hind* II fragmentation pattern to A7 and to no other *Alu* I fragment (Table 3). The alternative combinations at this junction, (130 + 190), (70 + 65) and (130 + 65) b.p., corresponding to A5, A9 and A8, respectively, can be ruled out. As will be shown in the next section, A5 and A8 are

located in the *Hae* III fragments Z3 and Z2 respectively (Fig. 4B). A9 is not cleaved by *Hind* II (Table 3).

Similarly A11 is the only *Alu* I fragment which corresponds to the combination (65 + 40) b.p. at the R3-R8 junction (Table 3). A12 overlaps the R8-R5 junction, (40 + 40) b.p. (Table 3). The alternative combination, (40 + 210) b.p., can be ruled out. The length corresponds to A7 but not the frag-

FIG. 3. Mass analysis of the DNA bands produced by complete digestion of ^{32}P -labeled ϕX RF with the *Arthrobacter luteus* restriction enzyme. Labeled ϕX RF was digested with *Alu* I and subjected to electrophoreses on a 7.5% polyacrylamide slab-gel. The Cerenkov counts of each band, which are proportional to the mass, were plotted against migration distance. The line $n = 1$ represents the line for one fragment per band. The parallel lines (---), $n = 2$, $n = 3$ and $n = 4$, are the calculated mass versus migration lines for bands with two, three and four fragments per band, respectively. Bands A1, A2 and A3 were not resolved on 7.5% gels, but the results on 3% gels indicated they had only one fragment per band.

mentation pattern (Table 3) and A7 has already been placed at the R4-R3 junction.

The one R7 fragment which is not cleaved by *Alu* I (Table 2) is R7A, since the overlapping fragment Z5 in the *Hae* III map (Fig. 4B) contains no *Alu* I cleavage site, as will be shown in the next section. Therefore R7B contains the *Alu* I cleavage site and A5 is the only *Alu* I fragment which corresponds to the combination (210 + 130 or 150) b.p. at the R5-R7B junction (Table 3).

At the R7B-R6B junction A6 corresponds to the combination (130 or 150 + 125) b.p. in length and cleavage pattern by *Hind* II; A7 can be ruled out, because of its cleavage pattern (Table 3). An alternative possibility is A1, (150 or 130 + 345 + 290 + 220) b.p., which is consistent with A1 in length and cleavage pattern by *Hind* II (Table 3). Three of the remaining combi-

nations, (150 or 130 + 345 + 290 + 125), (150 or 130 + 345 + 290 + 35) and (150 or 130 + 345 + 290 + 65) b.p., corresponding in length to A2 do not fit the cleavage pattern of A2 by *Hind* II given in Table 3. One remaining combination, (150 or 130 + 220) b.p., corresponding in length to A5 or A4 can be ruled out since A5 was already placed at the R5-R7B junction and A4 is not cleaved by *Hind* II (Table 3). The final remaining combinations, (150 or 130 + 35) and (150 or 130 + 65) b.p., correspond in length and cleavage pattern to A8 (Table 3). However, A8 can be ruled out since A8 is located in the fragment Z2 in the *Hae* III map (Fig. 4B).

Following A6, A1 is the only *Alu* I fragment which fits the R6B-R7A junction. The combination (220 + 290 + 345 + 150)

TABLE 1
LENGTH ESTIMATES OF ϕX RF FRAGMENTS PRODUCED BY CLEAVAGE WITH THE RESTRICTION ENDONUCLEASE FROM *Arthrobacter luteus*

<i>Alu</i> I fragment	Length in base pairs	Percentage of the total genome
A1	1100	19.9
A2	850	15.4
A3	700	12.7
A4	390	7.1
A5	350	6.3
A6	275	5.0
A7.1		
2	250 ^a	4.6 ^b
3		
A8	200	3.6
A9	140	2.5
A10	115	2.1
A11	105	1.9
A12.1		
2		
3	85 ^a	1.5 ^b
4		
A13	50	0.9
A14	40	0.7
A15.1		
2	33 ^a	0.6 ^b
A16	ca.28	0.5
A17	ca.24	0.4
Totals	5523	100%

^a Average length of the fragments.

^b Average percentage of the fragments.

TABLE 2
ANALYSIS OF SUBFRAGMENTS PRODUCED BY *Alu* I DIGESTION OF *Hind* II FRAGMENTS OF ϕ X RF DNA

<i>Hind</i> II fragment	<i>Alu</i> I partial digestion products	<i>Alu</i> I complete digestion products	Estimated fragment length (number of base pairs)	Linkage
R1	R1Ap1	R1A1	1000	150-80-50-755
			810	
	R1Ap2	R1A2	755	
			235	
	R1Ap3	A12	150	
			130	
R2	R2Ap1	A13	80	580-140-45
			50	
	R2Ap2	R2A2	760	
			725	
			580	
			190	
R3	R3Ap1	A9	140	190-390-65
			45	
	R3Ap2	R3A2	670	
			590	
			460	
			390	
R4	R4Ap1	R3A1	190	130-40-250-70
			65	
	R4Ap2	A7	510	
			440	
	R4Ap3	R4A2	310	
			250	
			165	
			130	
R5	R6Ap1	R4A2	70	40- $\begin{pmatrix} 85 \\ 33 \\ 28 \end{pmatrix}$ -210 ^a
			40	
	R6Ap2	A14	400	
			210	
			85	
			40	
			33	
			ca. 28	
R6 ^b	R6Ap1	A16 (or A17)	345	1 R6 not cleaved ^c 1 R6: 220-125 1 R6: 35-250-65
			320	
	R6Ap2	A7	280	
			250	
			220	
			125	
			65	
			35	
R7 ^a	R7A1	R6A1	295	1 R7 not cleaved ^c 1 R7: 150-130
			150	
			130	
R8	R8A1, R8A2	R7A2	205	40-120-40
			160	
			120	
			40 ^c	

TABLE 2—Continued

<i>Hind</i> II fragment	<i>Alu</i> I partial digestion products	<i>Alu</i> I complete digestion products	Estimated fragment length (number of base pairs)	Linkage
R9			155	
		R9A1	95	
		R9A2	50	
R10			80	

^a The 40 b.p. and 210 b.p. fragments have been placed terminally. They correspond in length to A14 and A8, respectively; however, A14 is located in R4 (see also the cleavage of Z2 by *Alu* I in Table 4) and A8 is cleaved by *Hind* II (Table 3).

^b R6 was digested as a mixture of R6A, R6B and R6C.

^c Measurement of the radioactivity of the digestion products from R6 and R7 showed that one R6 and one R7 were not cleaved.

^d R7 was digested as a mixture of R7A and R7B.

^e Radioactivity measurement indicated that this band was a doublet.

b.p. corresponds to A1 in length and *Hind* II cleavage pattern (Table 3). The alternative combinations at the R6B–R7A junction, following A6 are (220 + 290 + 35) and (220 + 290 + 65) b.p. which do not correspond in length to any of the *Alu* I fragments.

Following A1, the only overlapping *Alu* I fragment at the R6A–R1 junction is A6 (125 + 150) b.p., which combination fits A6 in length and *Hind* II fragmentation (Table 3). The alternative combination, (125 + 755) b.p., at this junction fits A2 in length, but not in fragmentation (Table 3).

A2 must be the fragment overlapping the R1–R9 junction, since the combination, (755 + 95) b.p., is the only one that corresponds in length and fragmentation pattern to the *Alu* I fragment A2 (Table 3). A3, (50 + 80 + 580) b.p., overlaps the R9–R10 and R10–R2 junction (see, however, the next section). This combination corresponds in length to A3. The fact that A3 is not cleaved by *Hind* II (Table 3) will be discussed in the next sections. The alternative combination, (50 + 80 + 45) b.p., does not correspond to the length or fragmentation pattern of any *Alu* fragment in Table 3. A12 overlaps the R2–R6C junction since the combination, (45 + 35) b.p., corresponds in length to A12. The alternative combination, (45 + 65) b.p., corresponding to A10 or A11 can be ruled out. A10 is not cleaved by *Hind* II (Table 3) and A11 has

been placed at the R3–R8 junction. Finally, the fragment A8 overlaps the R6C–R4 junction: The combination, (65 + 130) b.p., agrees with the length and fragmentation pattern of A8 (Table 3), and the position of A8 in the fragment Z2 of the *Hae* III map (Fig. 4B).

The partial *Alu* I map presented in Fig. 4A accommodates 21 of the 23 fragments that can be obtained by direct digestion of ϕ X RF. In order to place the remaining small fragments A15 and A17 and to confirm and extend the *Alu* I map, *Hae* III–*Alu* I and reciprocal digestions were performed as described in the next section.

Order of *Alu* I Fragments by Redigestion of *Hae* III Fragments of ϕ X RF by *Alu* I Enzyme

H. aegyptius cleaves ϕ X RF into 11 fragments which have been ordered by Lee and Sinsheimer (1974a). Table 4 shows the cleavage products obtained after redigestion of ³²P-labeled *Hae* III fragments Z1–Z10 by *Alu* I. Most of these redigestions yielded terminal *Hae*–*Alu* subfragments, and partials were only obtained with fragments Z1 and Z4 in which cases linkage could be established.

The *Alu* I cleavage sites from Table 4 are presented in the *Hae* III map of ϕ X RF in Fig. 4B. *Alu* I cleavage sites in the *Hae* III map which could be matched with unambiguous cleavage sites in Fig. 4A were

FIG. 4. (A), *Alu* I cleavage map of ϕ X RF showing the partial order of *Alu* I fragments A1–A16. The upper part of this figure represents the order of the *Hind* II fragments R1–R10 of ϕ X RF (Lee and Sinsheimer, 1974a) and the alternative positions of *Hind*–*Alu* subfragments within each R fragment (Table 2). (B), *Alu* I cleavage map of ϕ X RF showing the partial order of *Alu* I fragments A1–A17. The upper part of this figure represents the order of the *Hae* III fragments Z1–Z10 of ϕ X RF (Lee and Sinsheimer, 1974a) and the alternative positions of *Hae*–*Alu* subfragments within each Z fragment (Table 4).

TABLE 3
ANALYSIS OF SUBFRAGMENTS PRODUCED BY *Hind* II
DIGESTION OF *Alu* I FRAGMENTS OF ϕ X RF DNA

<i>Alu</i> I fragment	<i>Hind</i> II complete digestion products	Estimated fragment length (number of base pairs)
A1		1100
	R6	350
	R7	300
	A1R1 ^a	210
	A1R2 ^b	150
A2		850
	A2R1	755
	A2R2	95
A3		700
A4		390
A5		350
A6	A5R1	210
	A5R2	130
		275
	A6R1	150
A7 ^c	A6R2	120
		250
	A7R1	180
	A7R2	60
A8		200
	A8R1	135
	A8R2	75
A9		140
A10		115
A11		105
	A11R1	65
	A11R2	45

^a A1R1 corresponds in length to R8, but R8 is cleaved by *Alu* I (Table 2).

^b A1R2 has the same length as R9, but R9 is cleaved by *Alu* I (Table 2).

^c A7 was digested as a mixture of the three A7's. From the measurement of radioactivity of the digestion products it could be shown that two A7's were not cleaved by *Hind* II.

placed accordingly, and the corresponding Z fragments were drawn as solid lines.

The cleavage site in Z9 corresponds to the cleavage site between A11 and A10 as will be shown in the next section. Therefore the cleavage site between A4 and A11 which has not been observed in Z6B or Z9 must be very close to the Z6B-Z9 junction. The cleavage site between A10 and A12 can be deduced from the fragmentation of Z3 (Table 4), although the corresponding frag-

ment was not found (see next section). The other cleavage sites in Z3 match unambiguous cleavage sites in Fig. 4A. The cleavage site in Z7 has been placed in alternative combinations, represented by dotted lines. From these data in combination with the alternative cleavage patterns in the partial *Alu* I map (Fig. 4A) it can be concluded that the order of the *Alu* I fragments is A6, A1.

Besides one matching cleavage site in Z1, two new cleavage sites were found in Z1. One is probably very close to the R9-R10 junction and might therefore have been unobserved in *Hind* II-*Alu* digests. The other must then be located in R9. The 50 b.p. *Hind*-*Alu* subfragment at this position might represent a partial. On account of their lengths the small fragments in Z1 presumably represent A15 and A16 (or A17), respectively.

From these data a complete *Alu* I map (Fig. 4B) can be constructed which accounts for all the *Alu* I fragments that have been found by direct cleavage of ϕ F RF by *Alu* I.

Redigestion of *Alu* I Fragments by *Hae* III

The results obtained by redigestion of *Alu* I fragments with *Hae* III (Table 5) confirm the final *Alu* I map shown in Fig. 4B.

The cleavage pattern of A3 confirms the terminal position of a small 30 b.p. fragment in Z2. The cleavage pattern of A6 (Table 5) confirms the presence of the cleavage site close to the Z3-Z7 junction, as was already suggested from the cleavage of Z7 by *Alu* I (Table 4).

The cleavage pattern of A10 (Table 5) indicates that A10 overlaps Z10 and identifies two small fragments of 10 and 30 b.p., respectively, on either side of Z10. The 30 b.p. fragment is located in Z3 since the position of the 10 b.p. fragment in Z9, adjacent to Z10 is consistent with one of the alternative position of the *Alu* I cleavage site in Z9 (Fig. 4B). The cleavage site between A4 and A11 close to the Z6B-Z9 junction is located in Z6B on account of the cleavage pattern of A11 with *Hae* III (Table 5).

TABLE 4
ANALYSIS OF SUBFRAGMENTS PRODUCED BY *Alu* I DIGESTION OF *Hae* III FRAGMENTS OF ϕ X RF DNA

<i>Hae</i> III fragment	<i>Alu</i> I partial digestion products	<i>Alu</i> I complete digestion products	Estimated fragment length (number of base pairs)	Linkage
Z1			1200	650-(33, 28)-650
		Z1A1, Z1 A2	650 ^a	
	Z1Ap1		45	
		A15	33	
		A16 (or A17)	ca. 28	
Z2			1050	
		2 A7's	250 ^a	
		A8	200	
		A9	140	
		A12	75	
		Z2A1	65 ^b	
		A14	40	
		Z2A2	30 ^b	
Z3			870	
		A5	350	
		Z3A1	260 ^c	290-80-50-190
		2 A12's	80 ^a	
		A15	33	
		A16 (or A17)	ca. 28	
		Z3A2 ^d		
Z4			600	
	Z4Ap1		370	
	Z4Ap2		330	
		Z4A1	290	
	Z4Ap3		245	
		Z4A2	190	1 Z6: not cleaved
	Z4Ap4		140	
		A12	80	
		A13	50	
Z5			320	
Z6 ^e			285 ^f	
		Z6A1	190	
		Z6A2	100	
Z7			230	
		Z7A1	220	
		Z7A2 ^g		
Z8			190	
Z9			115	
		Z9A1	105	
		Z9A2	10	
Z10			73	

^a Radioactivity measurements indicated that these bands were doublets.

^b The terminal positions of the 65 b.p. and 30 b.p. fragments are consistent with the cleavage pattern of R4 by *Alu* I (Table 2) and that of A3 by *Hae* III (Table 5), respectively.

^c The fragment of 260 b.p. corresponds in length to A6, but A6 is cleaved by *Alu* I (Table 5). Furthermore, the length corresponds to A7, but two A7's are located in Z2 and one A7 is cleaved by *Hae* III (Table 5).

^d Z3A2 was not found. However, this fragment was identified as a 30 b.p. fragment from the *Hae* III digest of A10 (Table 5).

^e Z6 was digested as a mixture of Z6A and Z6B.

^f Measurement of the radioactivity of the bands showed that one Z6 was not cleaved.

^g Z7A2 was not found. However, the position of this *Alu* I cleavage site is consistent with the cleavage of A6 by *Hae* III (Table 5).

TABLE 5
ANALYSIS OF SUBFRAGMENTS PRODUCED BY *Hae* III
DIGESTION OF *Alu* I FRAGMENTS OF ϕ X RF DNA

<i>Alu</i> I fragment	<i>Hae</i> III complete digestion products	Estimated fragment length (number of base pairs)
A1		1100
	Z5	320
	A1Z1 ^a	285
	A1Z2 ^b	230
A2	Z8	190
		850
	A2Z1	680
A3	A2Z2	180
		700
	A3Z1	650
A4	A3Z2	35
		390
	A4Z1	285
A6	A4Z2	100
		275
	A6Z1	260
A7 ^d	A6Z2 ^c	250 ^e
		180
	A7Z1	60
A8	A7Z2	200
A9		140
A10		115
	Z10	75
	A10Z1	30
	A10Z2	10
A11		105
	A11Z1	100
	A11Z2 ^f	

^a A1Z1 corresponds to Z6 in length, but Z6 is not located in the Z5–Z8 region of the *Hae* III cleavage map (Lee and Sinsheimer, 1974a).

^b A1Z2 corresponds in length to Z7, but Z7 is cleaved by *Alu* I (Table 4).

^c The small fragment A6Z2 was not found. See, however, Table 4, footnote *g*.

^d A7 was digested as a mixture of the three A7's.

^e Radioactivity measurements indicated that two A7's were not cleaved by *Hae* III.

^f The small fragment A11Z2 was not found. See text.

DISCUSSION

The *Alu* I cleavage map of the circular ϕ X174 genome and alignment of this map with the *Hind* II and *Hae* III cleavage maps (Lee and Sinsheimer, 1974a) are shown in Fig. 4. The *Alu* I cleavage map is based on

the analysis of *Alu* I digestion products of *Hind* II and *Hae* III fragments and reciprocal digestion of *Alu* I fragments with *Hind* II and *Hae* III enzymes.

The relative positions of the clustered small *Alu* I fragments A12, A15 and A16 (or A17) adjacent to A5 and that of A15 and A16 (or A17) adjacent to A2 remain to be established. The fact that R10 is not cleaved by *Alu* I (Table 2) is consistent with the position of one *Alu* I cleavage site in close proximity to the R9–R10 junction. For reasons that are not understood, *Hind* II failed to cleave A3 (Table 3). However, it could be shown, by using hydroxyapatite chromatography (unpublished experiments), that ³²P-labeled A3 hybridized to R10 and R2 in solution.

According to the size estimates of the products obtained from the digestion of *Hae* III fragment Z1 with *Alu* I, the size of Z1 should be approximately 1350 base pairs. This is 150 base pairs more than has been estimated by Lee and Sinsheimer (1974a), but consistent with their redigestion data of Z1 with the restriction enzymes from *H. parainfluenzae* and *H. influenzae*.

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