Volume 3 no.8 August 1976 **Nucleic Acids Research**

# Cleavage map of bacteriophage ϕX174 RF DNA by restriction enzymes

P.D. Baas*, G.P.H. van Heusden, J.M. Vereijken, P.J. Weisbeek and H.S. Jansz

Institute for Molecular Biology, Laboratory for Physiological Chemistry and Department of Molecular Cell Biology, State University, Utrecht, The Netherlands

Received 17 May 1976

ABSTRACT

ϕX RF DNA was cleaved by restriction enzymes from Haemophilus influenzae Rf (Hinf I) and Haemophilus haemolyticus (Hha I). Twenty one fragments of approximately 25 to 730 base pairs were produced by Hinf I and seventeen fragments of approximately 40 to 1560 base pairs by Hha I. The order of these fragments has been established by digestion of Haemophilus aegyptius (Hae III) and Arthrobacter luteus (Alu I) endonuclease fragments of ϕX RF with Hinf I and Hha I.

By this method of reciprocal digestion a detailed cleavage map of ϕX RF DNA was constructed, which includes also the previously determined Hind II, Hae III and Alu I cleavage maps of ϕX 174 RF DNA (1, 2). Moreover, 28 conditional lethal mutants of bacteriophage ϕX174 were placed in this map using the genetic fragment assay (3).

## INTRODUCTION

Restriction endonucleases, which hydrolyze double stranded DNA at specific sites, have been isolated from a number of bacterial strains. Cleavage maps of ϕX RF DNA obtained with different restriction enzymes Hind II, Hae III, Hpa, Hap, Hin HI and Alu I, are already known (1-4). These cleavage maps have been used in a number of interesting analyses of ϕX DNA such as the study of ϕX DNA replication (5-9), the determination of the position of the RNA polymerase binding sites on ϕX RF DNA (10), the determination of the position of the only methylcytosine in ϕX DNA (11), the correlation

Abbreviations used: ϕX RF, double stranded circular replicative form DNA of ϕX174; ϕX RFI, supercoiled ϕX RF with both strands closed; ϕX RF II, nicked ϕX RF I; Hind II, Hae III, Alu I, Hha I, Hinf I, Hpa, Hap, Hin HI, are restriction endonucleases from H. influenzae Rd, H. aegyptius, Arthrobacter luteus, H. haemolyticus, H. influenzae Rf, H. parainfluenzae, H. aphrophilus, H. influenzae HI respectively; Z, R, A, H and F are restriction endonuclease fragments of ϕX RF DNA produced by Hae III, Hind II, Alu I, Hha I and Hinf I respectively. These fragments have been numbered according to their mobility as determined by gel electrophoresis, starting with the slowest e.g. Z1, Z2, Z3 etc.; b.p., base pairs; CAM, chloroamphenicol.

between the physical and genetic map of ϕX DNA (3), the induction of mutants in specific regions of the ϕX genome (12) and in ϕX DNA sequence studies (13, 14).

For further elucidation of these and other interesting functions of the ϕX genome and complete nucleotide sequence analysis the cleavage of ϕX RF into small fragments, 50 to 100 nucleotides in length is desirable which requires additional restriction endonucleases with unique cleavage specificity.

In this communication we describe the fragmentation of ϕX RF DNA by two other restriction enzymes. The endonuclease from Haemophilus influenzae Rf (Hinf I) cleaves ϕX RF DNA into twenty one fragments, 25 to 730 base pairs in length and the endonuclease from Haemophilus haemolyticus (Hha I) cleaves ϕX RF DNA into seventeen fragments, 40 to 1560 base pairs in length.

Hinf I has been discovered by C.A. Hutchison III and B.G. Barrel (personal communication) and Hha I by R.J. Roberts, P.A. Meyers, A. Morrison and K. Murray (15).

The Hinf I and Hha I fragments have been ordered and aligned with the Hae III, Alu I and Hind II cleavage maps of ϕX RF (2). By partial and complete digestions of Hae III fragments with Hinf I and Hha I endonucleases, followed by length analysis of the resultant products the positions of most of the Hinf I and Hha I cleavage sites could be established. The remaining sites could be deduced from the fragmentation pattern of overlapping Alu I fragments by Hinf I and Hha I endonucleases.
The complete cleavage map was correlated with the genetic map of ϕX.

When this work was in progress we were notified that in the MRC Laboratory of Molecular Biology in Cambridge, the relative order of the Hha I endonuclease fragments of ϕX174 was established using a completely different method of approach (16). The proposed order of the fragments agrees with that presented in this paper except for one additional fragment H14 (Fig. 3).

## MATERIALS AND METHODS

Isolation of uniformly labeled ($^{32}$P) ϕX RF I. E. coli C, grown in low phosphate containing medium, was infected with wild type ϕX in the presence of 40 µg CAM/ml. Carrier-free ($^{32}$P)$H_3PO_4$ (The Radiochemical Centre Ltd, Amersham, UK), 20 µCi/ml, was added 5 min after infection. Pure RF I was isolated as described by Vereijken et al. (2).

Preparation of restriction endonucleases. The preparation of the res-

triction endonucleases, Hae III and Alu I, was described previously (2). H. influenzae Rf and H. haemolyticus restriction enzymes were isolated in the same way according to the method described by Takanami and Kojo (17) and Takanami (18).

Preparation and analysis of endonuclease cleavage fragments. Digestions of ($^{32}$P) φX RF DNA and restriction fragments with other restriction endonucleases were carried out in 50 mM NaCl, 6.6 mMol Tris (pH 7.5) and 6.6 mMol $MgCl_2$ for 3 h at 37°. The required amount of restriction endoclease was determined in pilot experiments. The reactions were stopped by adding EDTA to 10 mMol and SDS to 1%. After incubation for 15 min at 37° sucrose and bromphenol blue tracking dye were added to 20% and 0.5% respectively. For preparative purposes restriction fragments were isolated by continuous electrophoresis on 5% polyacrylamide gels in cylindrical plexiglas tubes (1 x 25 cm) as described by Lee and Sinsheimer (19). The fragments were concentrated by BND cellulose chromatography (20) and isopropanol precipitation. φX DNA restriction fragments were redigested with appropriate amounts of other restriction enzymes and subjected to electrophoresis on 2 mm thick x 18 cm wide x 40 cm long slab gels of varying polyacrylamide concentrations (2). On the same gel also the undigested fragment as a control for purity and the total digest of ($^{32}$P) φX RF with the restriction enzymes in question were run. These digests were used for calibration of the gels. After autoradiography the radioactivity of the bands were determined by measuring the Cerenkov radiation of excised gel fragments in the scintillation counter. In a few experiments with non radioactive fragments the DNA was stained by emerging the gels in a solution of electrophoresis buffer containing 1 μg/ml ethidiumbromide.

The localization of conditional lethal mutants of bacteriophage φX174 on restriction enzyme fragments was described previously (3, 12).

## RESULTS

Number and molecular size of Hinf I and Hha I fragments of φX RF. Cleavage of ($^{32}$P) φX RF with Hinf I and Hha I endonucleases and subsequent electrophoresis on 5% polyacrylamide gels yielded 14 and 17 radioactive bands respectively (Fig. 1). The molecular sizes (number of base pairs) of the Hinf I and Hha I fragments were derived from the mobilities of the bands as compared with that of the Hae III fragments, the sizes of which have been well established (1, 2). Lee and Sinsheimer (1) have used for the calibration of their gels data of Maniatis, Jeffrey and Van de Sande (21),

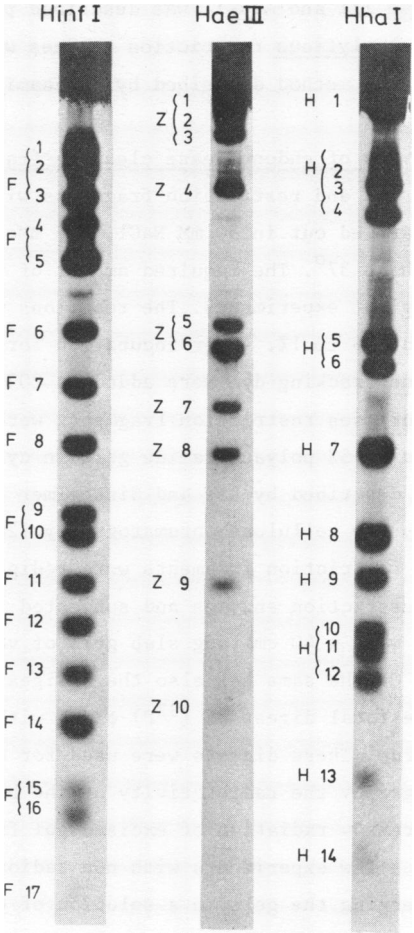


Figure 1:

Autoradiograms of fragmentation patterns of $^{32}$P-labeled ϕX RF DNA, digested with the restriction endonucleases from H. influenzae Rf, H. aegyptius and H. haemolyticus. The Hinf I, Hae III and Hha I digests comigrated in a 5% polyacrylamide slab gel.

who used as standards synthetic duplex DNA's which chain lengths were determined by chemical analysis (22). For the larger fragments this was performed on 3% polyacrylamide gels and for the smaller fragments on 6% polyacrylamide gels (up to 300 b.p.). From the relative yield of each band from uniformly labeled ($^{32}$P) ϕX RF it was concluded that the bands H5, H7 and H8 from the Hha I digest were twofold (Fig. 2a). Similarly band F5 from

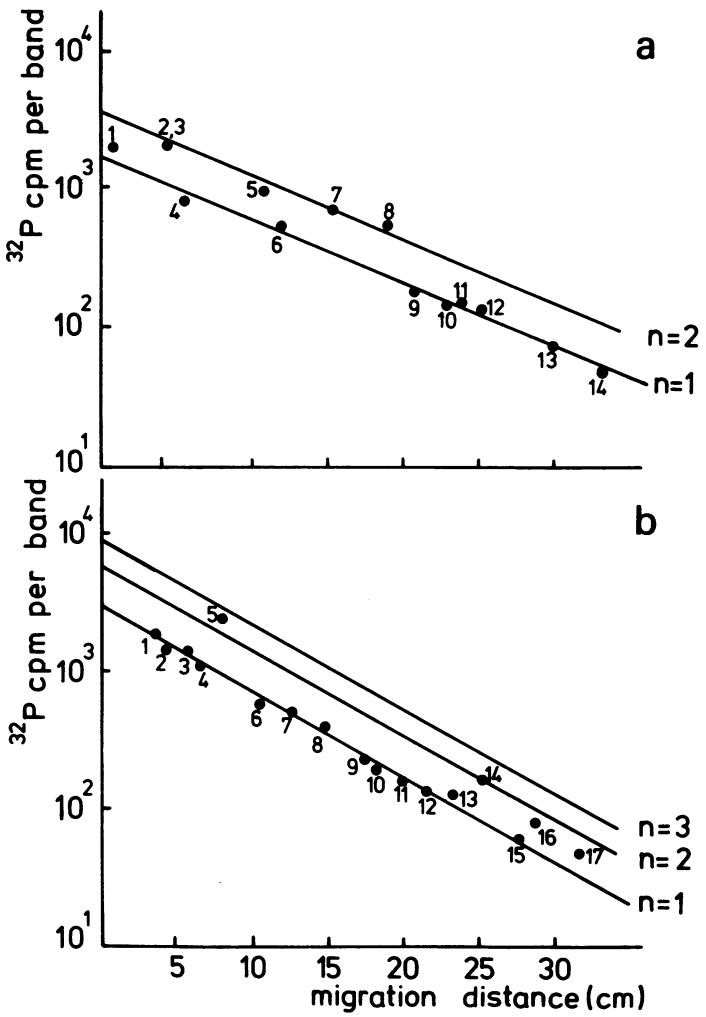


Figure 2:

Mass analysis of the DNA bands produced by complete digestion of $^{32}$P-labeled ϕX RF with the Haemophilus haemolyticus (a) and the Haemophilus influenzae Rf restriction enzyme (b). Labeled ϕX RF was digested with Hha I and Hinf I and subjected to electrophoresis on a 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 and n = 3 are the calculated mass versus migration lines for bands with two and three fragments per band respectively. Bands $H_2$ and $H_3$ (a) were not resolved on 5% gels and the radioactivity of these bands was measured together.

the Hinf I digest was threefold and the bands F14 and F16 were twofold (Fig. 2b). Tables 1 and 2 summarize the molecular size estimates as well as the fraction of the φX genome of each fragment for Hinf I and Hha I respectively.

TABLE 1

Length estimates of φX RF fragments produced by cleavage with the restriction endonuclease from Haemophilus influenzae Rf

| Hinf I fragment | Length in base pairs | Percentage of the total genome |
|---|---|---|
| F1 | 730 | 13.6 |
| F2 | 690 | 12.8 |
| F3 | 560 | 10.4 |
| F4 | 500 | 9.3 |
| F5.a | | |
| F5.b | 420-430 | 7.8-8.0 |
| F5.c | | |
| F6 | 310 | 5.8 |
| F7 | 245 | 4.6 |
| F8 | 196 | 3.7 |
| F9 | 149 | 2.8 |
| F10 | 138 | 2.6 |
| F11 | 116 | 2.2 |
| F12 | 100 | 1.8 |
| F13 | 82 | 1.5 |
| F14.a | 66 | 1.2 |
| F14.b | | |
| F15 | 50 | 0.9 |
| F16.a | 42 | 0.8 |
| F16.b | | |
| F17 | ~25 | 0.5 |
| | 5377 | 100.1% |

TABLE 2

Length estimates of φX RF fragments produced by cleavage with the restriction endonuclease from Haemophilus haemolyticus

| Hha I fragment | Length base pairs | Percentage of the total genome |
|---|---|---|
| H1 | 1560* | 29.0 |
| H2 | 635 | 11.8 |
| H3 | 615 | 11.5 |
| H4 | 525 | 9.8 |
| H5.a | 300 | 5.6 |
| H5.b | | |
| H6 | 270 | 5.0 |
| H7.a | 194 | 3.6 |
| H7.b | | |
| H8.a | 142 | 2.6 |
| H8.b | | |
| H9 | 123 | 2.3 |
| H10 | 102 | 1.9 |
| H11 | 94 | 1.8 |
| H12 | 85 | 1.5 |
| H13 | 58 | 1.0 |
| H14 | 40 | 0.7 |
| | 5379 | 99.9% |

The figures presented are the average of eight independent determinations.
* The length of the H1 fragment was determined by summing the digestion products of the relevant Hae III fragments by Hha I.

Complete Hinf I fragments inside Hae III fragments and partial fragment map. The general approach for ordering the Hinf I fragments was the partial and complete digestion of $^{32}$P-labeled Hae III fragments with Hinf I followed by size estimates of the resultant products on 5% polyacrylamide slab gels. When a Hae III fragment receives one cut by a second restriction enzyme, it should yield two terminal subfragments, if it receives two cuts it should yield two terminal subfragments and one complete fragment etc. Determination of the length of the partial digestion products reveals in some cases also information about the linkage of the fragments within the Hae III fragments. The results of the Hinf I digestions of Hae III fragments are summarized in Table 3.

From the data in Table 3 and the known Hae III fragment map the partial order of the Hinf I fragments could be deduced as follows. The order of the Z-fragments (Fig. 3) is Z2-Z6b-Z6a-Z9-Z10-Z3-Z7-Z5-Z8-Z4-Z1.

Complete Hinf I fragments occur inside the underlined Z-fragments (Table 3) which establishes the partial order: ---(F5, F10-F15, F12-F14)---(F6, F9, F16, F13-F17-F16)---(F5, F7, F8, F11)---.

This leaves the following F-fragments (Table 1) for the dotted regions,

TABLE 3

Analysis of subfragments produced by Hinf I digestion of Hae III fragments of ϕX RF DNA

| Hae III fragment | Hinf I partial digestion products | Hinf I complete digestion products | Estimated fragment length (number of base pairs) | Linkage |
|---|---|---|---|---|
| Z2 | | | 1050 | 240-(410,138-50,99-68)-57 |
| | Z2Fp1 | | 680 | |
| | Z2Fp2 | | 620 | |
| | Z2Fp3 | | 580 | |
| | Z2Fp4 | | 520 | |
| | Z2Fp5 | | 470 | |
| | | F5 | 410 | |
| | | Z2F1 | 240[a] | |
| | Z2Fp6 | | 186 | 138-50 |
| | Z2Fp7 | | 164 | 99-68 |
| | | F10 | 138 | |
| | | F12 | 99 | |
| | | F14 | 68 | |
| | | Z2F2 | 57 | |
| | | F15 | 50 | |
| Z6b | | | 285 | |
| Z6a | | | 290 | 225-64 |
| | | Z6aF1 | 225 | |
| | | Z6aF2 | 64 | |
| Z9 | | | 115 | |
| Z10 | | | 73 | |
| Z3 | | | 870 | 180-(315,148,43,83-25-43)-45 |
| | Z3Fp1 | | 720 | |
| | Z3Fp2 | | 690 | |
| | Z3Fp3 | | 645 | |
| | Z3Fp4 | | 580 | |
| | Z3Fp5 | | 530 | |
| | Z3Fp6 | | 490 | |
| | Z3Fp7 | | 380 | |
| | Z3Fp8 | | 340 | |
| | | F6 | 315 | |
| | Z3Fp9 | | 235 | |
| | Z3Fp10 | | 225 | |
| | | Z3F1 | 180 | |
| | | F9 | 148 | |
| | Z3Fp11 | | 120 | |
| | Z3Fp12 | | 104 | 83-25 |
| | | F13 | 83 | |
| | Z3Fp13 | | 67 | 25-43 |
| | | Z3F2 | 45 | |
| | | 2 F16's | 43[b] | |
| | | F17 | 25 | |
| Z7 | | | 230 | |
| Z5 | | | 320 | |
| Z8 | | | 190 | 136-53 |
| | | Z8F1 | 136 | |
| | | Z8F2 | 53 | |
| Z4 | | | 600 | |
| Z1 | | | 1350 | 250-(415,245,196,116)-114 |
| | | F5 | 415 | |
| | | Z1F1 | 250[c] | |
| | | F7 | 245 | |
| | | F8 | 196 | |
| | | F11 | 116 | |
| | | Z1F2 | 114[c] | |

a) The 240 b.p. fragment has been placed terminally. It corresponds in length to F7, however, F7 is located in Z1.
b) Radioactivity measurement indicated that this band was a doublet.
c) The choice between Z1F1 and F7 (or F11 and Z1F2) is arbitrary.

i.e. fragments which overlap the Hae III cleavage sites in ϕX RF DNA: F1 (730 b.p.), F2 (690 b.p.), F3 (560 b.p.), F4 (500 b.p.), F5 (ca 425 b.p.) and F14 (66 b.p.).

From the linkage data in Table 3 the following alternative lengths can be derived for the dotted regions:

$$- \begin{matrix}242\\57\end{matrix} - (F5, F10\text{-}F15, F12\text{-}F14) - \begin{matrix}242\\57\end{matrix} - 285 - \begin{matrix}225 - 64\\64 \quad 225\end{matrix} - 115 - 73 - \begin{matrix}180\\45\end{matrix} -$$

$$- (F6, F9, F16, F13\text{-}F17\text{-}F16) - \begin{matrix}180\\45\end{matrix} - 230 - 320 - \begin{matrix}136 - 53\\53 - 136\end{matrix} - 600 - \begin{matrix}250\\114\end{matrix} -$$

$$- (F5, F7, F8, F11) - \begin{matrix}250\\114\end{matrix} -.$$

Therefore the order of the Hinf I fragments should be:-F4-(F5, F10-F15, F12-F14)-F3-F5-(F6, F9, F16, F13-F17-F16)-F1-F14-F2-(F5, F7, F8, F11)-F4-.

In order to place F14 (66 b.p.) one extra Hinf I cut close to the Z8-Z4 junction must be assumed. This is confirmed by the digestion pattern of A1 by Hinf I (Table 4).

Order of the Hinf I fragments derived from fragmentation patterns of Alu I fragments. In a previous paper (2) we have located the positions of the cleavage sites of the Hind II, Alu I and Hae III restriction enzymes on ϕX RF with respect to each other (Figs. 3 and 4). This makes it possible to confirm and extend the partial F-fragment map by digestion of Alu I fragments that overlap the unresolved parts of the map with Hinf I endonuclease. Therefore overlapping Alu I fragments were digested with Hinf I followed by size estimates of the resultant products on 5% polyacrylamide gels. The results are shown in Table 4.

The order of the fragments (F6, F9, F16, F13-F17-F16) inside Z3 follows from the digestion pattern of the adjacent fragments A5 and A6 by Hinf I (Table 4):

$$- \begin{matrix}107 - 230\\230 - 107\end{matrix} - 35 - F13 - F17 - F16 - F16 - 52 -.$$

The small fragments F13-F17-F16-F16 must be located at the right side of Z3 since A6 is at the right side of Z3 (Fig. 3). The 52 b.p. fragment must be at the right side of A6, since A6 overlaps the Z3-Z7 junction with 10 b.p. (Figs. 3 and 4) and Z3 yields a 45 b.p. terminal fragment after Hinf I digestion (Table 3). F9 (149 b.p.) overlaps the A5-A6 junction in the combination 107 + 35 b.p. and F6 (310 b.p.) must be located at the left part of Z3. This established the order -F5-F6-F9-F13-F17-F16-F16-F1-.

The order of the fragments (F5, F7, F8, F11) inside Z1 follows from the fragmentation pattern of A2 and A3 (Table 4) -300-F11-F7-172- and

TABLE 4

Analysis of subfragments produced by Hinf I digestion of Alu I fragments of ϕX RF DNA

| Alu I fragment | Hinf I partial digestion products | Hinf I complete digestion products | Estimated fragment length (number of base pairs) | Linkage |
|---|---|---|---|---|
| A1 | | | 1100 | ∿700-68-275 |
| | | A1F1 | ∿700 | |
| | | A1F2 | 275 | |
| | | F14 | 68 | |
| A2 | | | 850 | 300-118-245-172 |
| | A2Fp1 | | 660 | 300-118-245 |
| | A2Fp2 | | 530 | 118-245-172 |
| | A2Fp3 | | 415 | 300-118-and/or 245-172 |
| | A2Fp4 | | 360 | 118-245 |
| | | A2F1 | 300 | |
| | | F7 | 245 | |
| | | A2F2 | 172 | |
| | | F11 | 118 | |
| A3 | | | 700 | 280-200-175 |
| | | A3F4 | 280 | |
| | | F8 | 200 | |
| | | A3F2 | 175 | |
| A5 | | | 350 | 230-107 |
| | | A5F1 | 230 | |
| | | A5F2 | 107 | |
| A6 | | | 275 | 35-83-25-42-42-52 |
| | A6Fp1 | | 220 | 35-83-25-42-42 |
| | A6Fp2 | | 180 | 35-83-25-42 |
| | A6Fp3 | | 157 | 25-42-42-52 |
| | A6Fp4 | | 143 | 35-83-25 |
| | A6Fp5 | | 135 | 42-42-52 |
| | A6Fp6 | | 119 | 35-83 |
| | A6Fp7 | | 109 | 83-25 |
| | A6Fp8 | | 93 | 42-52 |
| | | F13 | 83 | |
| | A6Fp9 | | 71 | 25-42 |
| | | A6F1 | 52 | |
| | | 2 F16's | 42[b] | |
| | | A6F2 | 35 | |
| | | F17 | ∿25 | |
| A7[c] | | | 250 | 1 A7 is not cleaved |
| | | A7cF1 | 240 | A7c: 240-10 |
| | | F12 | 102 | |
| | | A7aF1 | 93 | A7a: 50-102-93 |
| | | A7aF2 | 50 | |
| | | A7cF2[a] | | |
| A8 | | | 200 | 103-50-50 |
| | | A8F1 | 103 | |
| | | F15, A8F2 | 50[b] | |

a) These fragments were not found, however, the presence of these fragments can be deduced from the digestion of overlapping restriction fragments.
b) Radioactivity measurement indicated that these bands were doublets.
c) A7 was digested as a mixture of A7a, A7b and A7c.

-175-F8-280-. F5 (ca 420 b.p.) overlaps the A2-A16-A15-A3 junctions (172 + 33 + 25 + 175 b.p.). This established the order (see previous section): -F2-F11-F7-F5-F8-F4-.

The order of the Hinf I fragments within Z2 (F5, F10-F15, F12-F14) follows from the fragmentation of the adjacent A7 and A8 by Hinf I (Table 4): -50-F12-93- and -50-F15-103-. This places F10 (93 + 50 b.p.) at the A7-A8 junction which establishes the order -F12-F10-F15-. In combination with the partial order from the previous section this indicates the order: -F14-F12-F10-F15-. Finally the large fragment F5 (ca 420 b.p.) must be located at the right side of Z2 to overlap the fragment A7b. The final order of the Hinf I fragments is: -F4-F14-F12-F10-F15-F5-F3-F5-F6-F9-F13-F17-F16-F16-F1-F14-F2-F11-F7-F8-F4- (Figs. 3 and 4).

Hha I cleavage map of ϕX. The order of the senteen Hha I fragments was determined in the same way as the order of the Hinf I fragments, by partial and complete digestion of Hae III and Alu I restriction fragments with the Hha I enzyme (data not shown).

The order of the Hha I fragments is: H2-H8-H1-H14-H7-H4-H12-H10-H13-H11-H9-H6-H5-H8-H7-H5-H3-H2 (Figs. 3 and 4).
The order of Hha I fragments is in agreement with the order proposed by Jeppesen et al. (16), except for one additional fragment H14. H14 is located in the Hae III fragment Z3. Table 5 shows the partial and complete digestion products of the Hae III fragment Z3 with Hha I.

TABLE 5.
Analysis of subfragments produced by Hha I digestion of the Hae III fragment Z3

| Hae III fragment | Hha I partial digestion products | Hha I complete digestion products | Estimated fragment length (number of base pairs) | Linkage |
|---|---|---|---|---|
| Z3 | | | 870 | 360-40-198-270 |
| | Z3Hp1 | | 600 | 360-40-198 |
| | Z3Hp2 | | 500 | 40-198-270 |
| | Z3Hp3 | | 460 | 198-270 |
| | Z3Hp4 | | 400 | 360-40 |
| | | Z3H1 | 360[a] | |
| | | Z3H2 | 270[b] | |
| | Z3Hp5 | | 235 | 40-198 |
| | | H7 | 198 | |
| | | H14 | 40 | |

a) The Z3H1 fragment (360 b.p.) is part of the H1 fragment.
b) The 270 b.p. fragment has been placed terminally. It corresponds in length to H6, however H6 is located in A1 (data not shown): the Z3H2 fragment is part of H4.

## DISCUSSION

The method used in this paper and the previous one (2) to construct the cleavage map of ϕX RF DNA was the digestion of restriction enzyme fragments with known positions on the ϕX genome. The Hinf I and Hha I maps were obtained from the digestion patterns of Hae III and Alu I fragments, the positions of which have been established previously (1, 2). The advantage

of this method is that the distances i.e. number of base pairs between the various endonuclease cleavage sites become accurately known during this work which allows the construction of a detailed ϕX RF cleavage map (Figs. 3 and 4). With respect to this map we want to emphasize the following

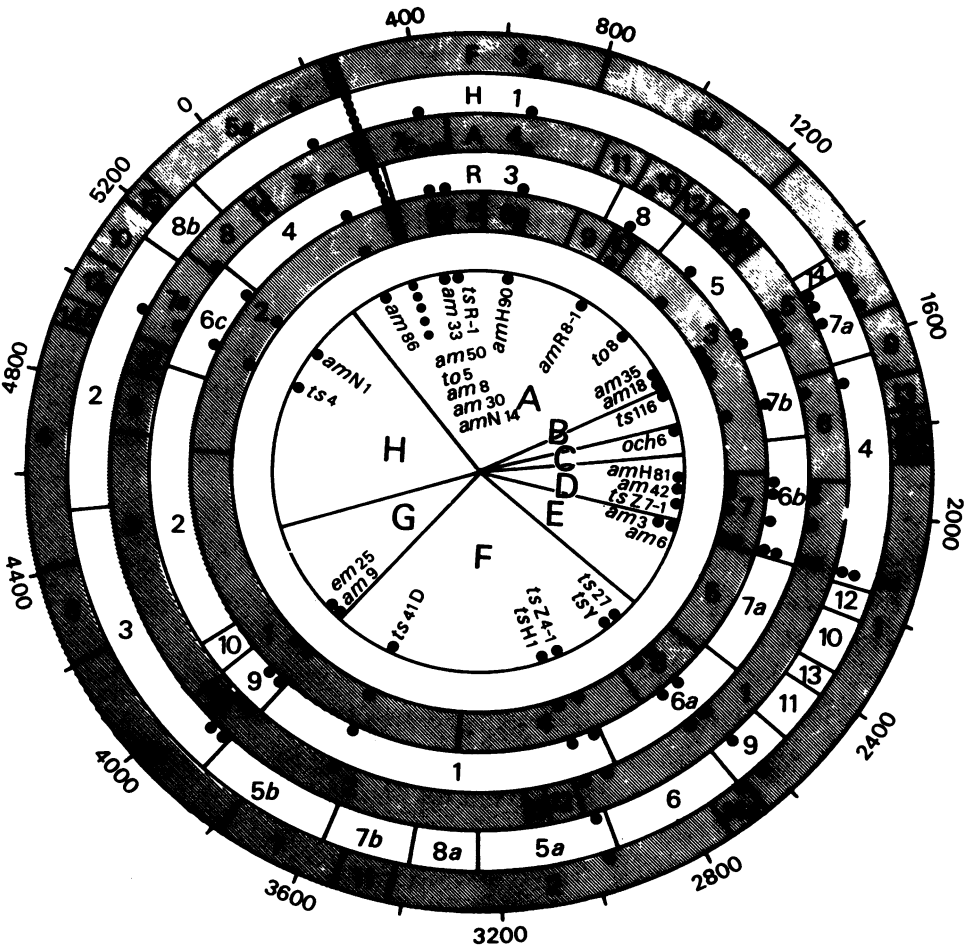


Figure 3:

A circular presentation of the cleavage map of ϕX RF DNA. From outside to inside the map represents the Hinf I, F-fragment map, the Hha I, H-fragment map, the Alu I, A-fragment map, the Hind II, R-fragment map and the Hae III, Z-fragment map. The inner circle represents a correlation between the physical and genetic map of ϕX. The different mutants were placed on the various restriction fragments and the borders between the different cistrons were determined as described by Weisbeek et al. (3). On the outer circle map distances in nucleotides starting at the gene A/H border are given (see also Fig. 4). The relative positions of A12, A15 and A16 (in R5) have not yet been established.

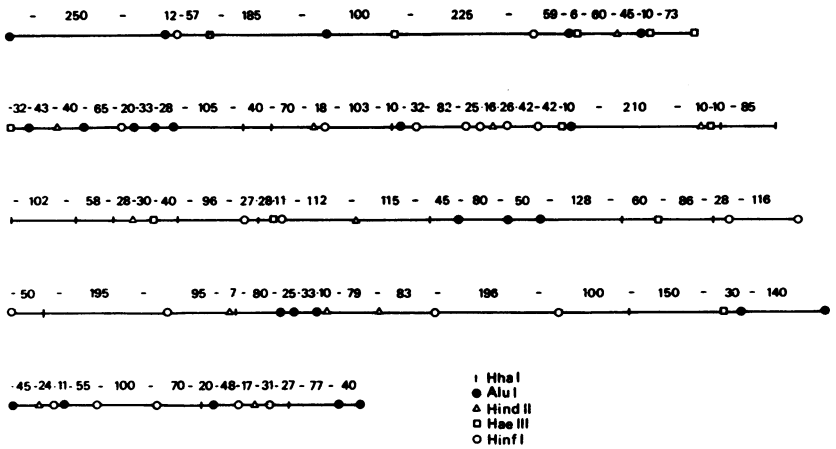


Figure 4:

A linear presentation of the cleavage map of ϕX RF DNA. This map starts at the A14/A7b cleavage site of Alu I and represents the map distances in nucleotides between the cleavages sites of Hha I, Alu I, Hind II, Hae III and Hinf I. The relative position of the Hae III and Hind II cleavage site Z2/Z6b and R4/R3 respectively, have not yet been established.

points. First, it is generally accepted to use for multiple and hard to separate bands the notation a for the slower fragment, b for the faster one, and so on. With respect to the multiple R6 and R7-fragments of the Hind II map we have followed the notation of Lee and Sinsheimer (1). In our laboratory we have not tried any further separation of the fragments in these bands. The Hae III digest of ϕX RF contains one double band, Z6. Z6a and Z6b could be separated using long gels containing ethyleneglycodiacrylate as a cross-linker instead of bisacrylamide, and their order has been determined (3, 9) which is the reverse of that presented by Lee and Sinsheimer (1). With regard to the other enzymes the notation a, b, c and d is completely arbitrary. We only know that the fastest A12 fragment is located in Z2.

Secondly, Vereijken et al. (2) were not able to solve the order of the small Alu I fragments 15 and 16 in R9. From the sequence data of Air et al. (14) and the known recognition site of Alu I, AGCT (23), we now know the order A16-A15 (clockwise) and their exact length of 25 and 33 b.p. respectively. From the work of Air et al. (14) the exact length of R10, 79 b.p. is also known. Similarly sequence dat (F. Sanger, personal communication) and the known recognition site of Hha I, GCGC (15) established the length

of H9, 123 b.p. Also from this sequence data and the recognition site of Hinf I, GAXTC (C.A. Hutchison III and B.G. Barrel, personal communication) the length of the fragment F14a, 66 b.p., could be verified.

Finally, we want to emphasize that, although most of the cleavage sites shown in Figs. 3 and 4 were determined, some of them were deduced. For the construction of the map we have used the following data:

1. digestion of all Hae III fragments with Alu I (2), with Hind II (1), with Hha I (unpublished results) and with Hinf I (this paper)
2. digestion of all the Hind II fragments with Alu I (2)
3. digestion of most of the Alu I fragments with Hind II and Hae III (2)
4. digestion of most of the Alu I fragments with Hinf I (this paper) and with Hha I (unpublished results)
5. double digestion of ϕX RF with Hae III and Hind II and with Hae III and Hha I (unpublished results)
6. ϕX DNA sequence data from Air et al. (14) and F. Sanger (personal communication).

The total length of the ϕX RF DNA amounts to 5357 b.p. This figure is obtained by summing up fragments ranging in size between 25 and 250 b.p. as determined from their relative mobilities on polyacrylamide gel electrophoresis. These lengths compare well (within 5%) with the lengths as determined by sequence analysis in those few cases where this comparison can be made. It is also in agreement with the number of nucleotides of the ϕX genome (5479 b.p.) that can be calculated from the pyrimidine tracts analyses of the whole viral (24) and the whole complementary (25) strand of ϕX DNA. Recently the total number of nucleotides of ϕX DNA has been determined on 4800 ± 160 using absolute physical-chemical methods (26). These figures are reasonably close to our figure considering the accuracies of the methods involved.

ACKNOWLEDGEMENTS

We acknowledge the help of Dr. Richard J. Roberts who kindly digested ϕX RF DNA with a number of restriction enzymes on our request. His results indicated that Hinf I and Hha I produced many fragments which encouraged us to undertake the present work. We also thank Dr. Roberts for sending us the bacterial strains for the preparation of Hinf I and Hha I.

We thank Prof. K.C. Winkler for the help in handling and growing H. haemolyticus.

We thank Mr. F. Heidekamp for the preparation of the restriction

endonuclease Hinf I and Dr. C.H. Monfoort for the preparation of Hha I. This work was supported in part by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

*Address: Institute for Molecular Biology, Transitorium 3, Padualaan 8, Utrecht, The Netherlands

REFERENCES

1. Lee, A.S. and Sinsheimer, R.L. (1974) Proc. Nat. Acad. Sci. USA 71, 2882-2886
2. Vereijken, J.M., van Mansfeld, A.D.M., Baas, P.D. and Jansz, H.S. (1975) Virology 68, 221-233
3. Weisbeek, P.J., Vereijken, J.M., Baas, P.D., Jansz, H.S. and van Arkel, G.A. (1976) Virology, in press
4. Hayashi, M.N. and Hayashi, M. (1974) J. Virol. 14, 1141-1151
5. Johnson, P.H. and Sinsheimer, R.L. (1974) J. Mol. Biol. 83, 47-61
6. Eisenberg, S. and Denhardt, D.T. (1974) Proc. Nat. Acad. Sci. USA 71, 984-988
7. Eisenberg, S. and Denhardt, D.T. (1974) Biochem. Biophys. Res. Commun. 61, 532-537
8. Godson, G.N. (1974) J. Mol. Biol. 90, 127-141
9. Baas, P.D., Jansz, H.S. and Sinsheimer, R.L. (1976) J. Mol. Biol. 102, 633-656
10. Chen, C.Y., Hutchison, C.A. III and Edgell, M.H. (1973) Nature New Biol. 243, 233-236
11. Lee, A.S. and Sinsheimer, R.L. (1974) J. Virol. 14, 872-877
12. Borrias, W.E., Wilschut, I.J.C., Vereijken, J.M., Weisbeek, P.J. and van Arkel, G.A. (1976) Virology 70, 195-197
13. Sanger, F. and Coulson, A.R. (1975) J. Mol. Biol. 94, 441-448
14. Air, G.M., Blackburn, E.H., Sanger, F. and Coulson, A.R. (1975) J. Mol. Biol. 96, 703-719
15. Roberts, R.J., Myers, P.A., Morrison, A. and Murray, K. (1976) J. Mol. Biol. 103, 199-208
16. Jeppesen, P.G.N., Sanders, L. and Slocombe, P.M. (1976) Nucleic Acid Res. 5, 1323-1340
17. Takanami, M. and Kojo, H. (1973) FEBS Lett. 29, 267-270
18. Takanami, M. (1973) FEBS Lett. 34, 318-322
19. Lee, A.S. and Sinsheimer, R.L. (1969) Anal. Biochem. 60, 640-644
20. Kiger, J.A. and Sinsheimer, R.L. (1969) J. Mol. Biol. 40, 467-490
21. Maniatis, T., Jeffrey, A. and van de Sande, H. (1975) Biochemistry 14, 3787-3794
22. Khorana, H.G., Agarwal, K.L., Buchi, H., Caruthers, M.H., Gupta, N.K., Kleppe, K., Kumar, A., Ohtsuka, E., Raj Bhandary, U.L., van de Sande, H., Sgaramella, V., Terao, T., Weber, H. and Yamada, R. (1972) J. Mol. Biol. 72, 209-217
23. Roberts, R.J., Myers, P.A., Morrison, A. and Murray, K. (1976) J. Mol. Biol. 102, 157-165
24. Hall, J.B. and Sinsheimer, R.L. (1963) J. Mol. Biol. 6, 115-127
25. Darby, G., Dumas, L.B. and Sinsheimer, R.L. (1970) J. Mol. Biol. 52, 227-238
26. Berkowitz, S.A. and Day, L.A. (1974) Biochemistry 13, 4825-4831