JOURNAL OF VIROLOGY, Sept. 1975, p. 674-684
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Vol. 16, No.3
Printed in U.S.A.

Endonuclease R·*Eco*RII Restriction of Bacteriophage f1 DNA In Vitro: Ordering of Genes V and VII, Location of an RNA Promotor for Gene VIII

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Received for publication 25 March 1975

Replicative form DNA of bacteriophage f1 was found to be sensitive in vitro to restriction by endonuclease R·*Eco*RII if the DNA was isolated from an *Escherichia coli* strain deficient in cytosine methylase activity. A similar observation was previously made with DNA from the closely related bacteriophage fd (S. Schlagman, S. Hattman, M. S. May, and L. Berger, submitted for publication). The two DNA fragments produced by the endo R·*Eco*RII digestion of f1 DNA were localized on the f1 cleavage map and their genetic content was determined. The polypeptides synthesized in a "coupled" transcription-translation system under the direction of each RII fragment were examined. The results of such experiments allow the ordering of genes V and VII and indicate the location of a RNA promoter for gene VIII.

f1, fd, and M13 are closely related, filamentous bacteriophage that infect male strains of *Escherichia coli* (16). None of these phage is sensitive in vivo to restriction specified by the *hsp*II locus (1, 2, 9). The genes responsible for the *hsp*II system are carried by at least two independently isolated drug-resistance transfer factors: R-15, sometimes referred to as RTF-2 (1, 32), and N-3 (28, 30). Recent in vivo and in vitro studies have indicated that the modification methylase specified by the *hsp*II locus methylates DNA sequences that are also methylated by an enzyme found in certain bacterial strains and specified by a chromosomal locus, *mec* (10, 22; S. Hattman, personal communication). Both enzymes methylate cytosine residues to form 5-methylcytosine (4, 8, 22). DNA sequence studies have demonstrated that the cytosine residues methylated by an enzyme isolated from an *E. coli* strain containing the R-15 plasmid are found within the DNA sequence restricted by the *hsp*II specified restriction endonuclease, endo R·*Eco*RII (4). (The nomenclature of Smith and Nathans [24] has been used.)

The single-stranded, circular viral DNA of bacteriophage fd contains between two to three 5-methylcytosine residues per genome if the phage has been grown in an N-3 plasmid containing *E. coli* strain that is either *mec*$^+$ or *mec*$^-$ (9, 10, 22). No detectable 5-methylcytosine is found if the phage has been grown in a *mec*$^-$ *E. coli* strain which lacks both the N-3 and R-15 plasmids (10, 22). Bacteriophage fd lacking 5-methylcytosine residues, however, is still not restricted in vivo by the *hsp*II restriction-modification system, i.e., fd grown in *mec*$^-$ *E. coli* plate with the same efficiency on *mec*$^+$, $r_{N\text{-}3}{}^+m_{N\text{-}3}{}^+$ as on *mec*$^+$ *E. coli* (10). Nevertheless, S. Schlagman, S. Hattman, M. S. May, and L. Berger (submitted for publication) have recently demonstrated that fd RFI (a covalently linked, circular, double-stranded DNA molecule) isolated from a *mec*$^-$ *E. coli* strain is restricted in vitro by endo R·*Eco*RII, whereas the *mec*$^+$ RFI is inert to the enzyme. (*Mec*$^+$ DNA is DNA isolated from a *mec*$^+$ *E. coli* strain whereas *mec*$^-$ DNA is DNA isolated from a *mec*$^-$ *E. coli* strain.)

There are known differences in the sensitivities to restriction between the closely related bacteriophage f1, fd, and M13 in vivo and in vitro, e.g., B restriction in vivo (1), the number of fragments produced by endo R·*Hae*III in vitro (C. A. van den Hondel and J. Schoenmakers, Eur. J. Biochem., in press; K. Horiuchi, G. F. Vovis, V. Enea, and N. D. Zinder, J. Mol. Biol., in press). Therefore, we have tested the sensitivity in vitro of *mec*$^-$ f1 RFI to restriction by endo R·*Eco*RII. We report here that, as is fd RFI, f1 RFI is sensitive to endo R·*Eco*RII restriction if the DNA is isolated from a *mec*$^-$ *E. coli* strain. We have mapped the location of the two endo R·*Eco*RII-specific restriction breaks in relation to the recently mapped f1 HaeIII fragments (Horiuchi et al., J. Mol. Biol., in

press) and have determined by marker rescue experiments which genes are contained on each RII fragment. The results of such experiments allow us to order genes V and VII, the order of which was previously ambiguous (15; L. B. Lyons, Ph.D. Thesis, The Rockefeller Univ., New York, 1971). Furthermore, preliminary studies with isolated RII restriction fragments in a "coupled" transcription-translation system (17) reveal the location of a RNA promotor for gene VIII, the gene which codes for the major coat protein (16).

MATERIALS AND METHODS

Bacteria. *E. coli* strains K37 (*Su*-1, $r_K^+m_K^+$), K38 (Su^-, $r_K^+m_K^+$), K143 (*Su*-3, $r_K^+m_K^+$), and K336B (Su^-, $r_B^+m_B^+$) are from this laboratory (15). The sources of the other bacterial strains are: *mec*$^-$ *E. coli* (F^+, *mec*$^-$, *endo*I$^-$, B_1^-, $r_K^-m_K^+$) from S. Hattman (10); *E. coli* RY-22 (F^+, *Str*^{r}, *Gal*$^-$, *endo*I$^-$, $r_B^+m_B^+$, R-15 [*Str*^{r}, *Sul*^{r}, $r_{RII}^+m_{RII}^+$]) of R. N. Yoshimori (Ph.D. Thesis, Univ. of California, San Francisco, 1971) from R. Roberts; *Hemophilus influenzae* strain Rd from H. O. Smith; *H. aegyptius* from R. Roberts.

Phage. Bacteriophage f1 and its mutants were isolated in this laboratory. The amber mutants, most of which have been previously described (15, 17), were classified by complementation tests (15, 21). The SB mutants used (SB sites are genetic sites which confer upon DNA sensitivity to restriction and modification by *E. coli* B, i.e., $r_B^+m_B^+$) and their genetic location have been described (15). The amber mutants of bacteriophage M13 (3H5, 7H1, 8H1) were obtained from D. Pratt (20, 21). The f1 and M13 amber mutants were grown on either *E. coli* strain K37 or K143 (see above).

Isolation of DNA. RFI of f1 was isolated by the modified procedure (Horiuchi et al., J. Mol. Biol., in press) of the method described by Model and Zinder (17). The isolation of ^{32}P-labeled f1 RFI (Horiuchi et al., J. Mol. Biol., in press) and f1 viral DNA (12) have been described. Simian virus 40 DNA was very generously provided by M. Mathews.

Isolation of restriction endonucleases. Endo R·*Eco*RII was isolated from *E. coli* strain RY-22 (see above) essentially by the method described by Yoshimori (Ph.D. Thesis). Endo R·*Hin*d was isolated from *H. influenzae* strain Rd (see above) as described by Smith and Wilcox (25) except that the nucleic acids were removed by precipitation with streptomycin sulfate (23). Endo R·*Hae*III was isolated from *H. aegyptius* (see above) as before (Horiuchi et al., J. Mol. Biol., in press).

Isolation of f1 RII-A and RII-B fragments. The ^{32}P-labeled *mec*$^-$ f1 RFI which had been digested with endo R·*Eco*RII (see legend to Fig. 1) was layered on a 1.8% agarose slab gel (40 by 15 by 0.3 cm). The running buffer is the same as described in the legend to Fig. 1 except that the ethidium bromide was omitted. The gel, connected to the upper reservoir by a Whatman 3MM paper wick, was run at 225 to 275 V. The fragments, located by radioautography of the wet gel, were extracted and concentrated as before (Horiuchi et al., in press).

Transfection. The procedure for preparing the heteroduplexes and transfecting the $CaCl_2$-treated *E. coli* (29) has been described (Horiuchi et al., in press).

Coupled transcription-translation system. The isolated f1 RII-A and RII-B fragments (see above) were added to the system described by Model and Zinder (17). Analysis was carried out on an exponential polyacrylamide gradient gel of the kind described by P. O'Farrell (J. Biol. Chem., in press) except that the acrylamide concentration ranged from 10 to 18% and 8 M urea was present.

RESULTS

Sensitivity of mec$^-$ f1 RFI to restriction by endo R·EcoRII. To determine whether f1 DNA which lacks methylated cytosine residues is sensitive in vitro to restriction by endo R·*Eco*RII, RFI was isolated from an *E. coli* strain deficient in cytosine methylase activity, *mec*$^-$ (10). The *mec*$^-$ RFI was incubated in the presence of endo R·*Eco*RII under conditions where simian virus 40 DNA, which reportedly has about 17 cleavage sites (4), is extensively restricted (Fig. 1a and b). Under these conditions, the *mec*$^-$ f1 RFI was broken into two fragments (Fig. 1e and f), whereas the *mec*$^+$ f1 RFI was inert to the enzyme (Fig. 1c and d). The larger fragment produced by the endo R·*Eco*RII digestion of *mec*$^-$ f1 RFI, RII-A, migrated slightly faster on an agarose slab gel than the full length, linear, double-stranded DNA molecule produced by the enzymatic cleavage of f1 RFI with endo R·*Hin*dII, i.e., RFIII/*Hin*dII (Horiuchi et al., in press) (Fig. 1f and g; this difference in mobility was more apparent with longer times of electrophoresis). The smaller fragment, RII-B, migrated slower than HaeIII-C (Fig. 1f and h), the 13% of f1 RFI size fragment produced by digesting f1 RFI with endo R·*Hae*III (Horiuchi et al., in press). No other fragments could be detected in the endo R·*Eco*RII digest of *mec*$^-$ f1 RFI even by electrophoretic analysis of a digest made with ^{32}P-labeled DNA on a 4% polyacrylamide gel (data not shown). Thus, as observed with fd (Schlagman et al., submitted for publication), f1 RFI, if isolated from an *E. coli* strain deficient in cytosine methylase activity, is sensitive in vitro to restriction by endo R·*Eco*RII.

The location of the endo R·EcoRII cleavage sites on the cleavage map of f1. Endo R·*Hae*III digestion of f1 RFI produces nine fragments which have been ordered so as to construct a circular map of the f1 genome (Horiuchi et al., in press). To determine the location of the two endo R·*Eco*RII breaks on

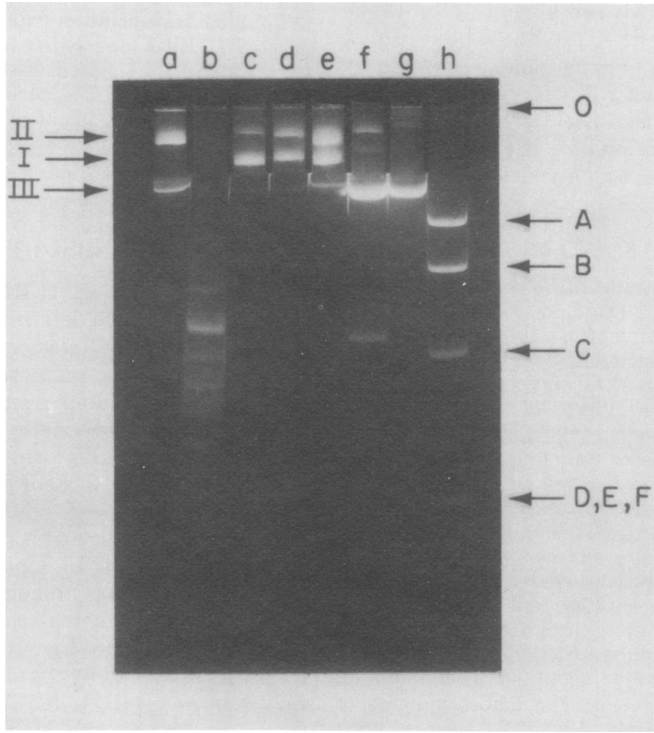

FIG. 1. *Endonuclease R·EcoRII digestion of mec⁻ and mec⁺ f1 RFI. The 50-μl endo R·EcoRII-containing reaction mixtures also contained 90 mM Tris-hydrochloride, pH 7.4, and 5 mM $MgCl_2$. Incubation was at 35 C for 17 h. Conditions for endo R·Hind and endo R·HaeIII digestion were as before (Horiuchi et al., in press). EDTA (40 mM), 0.08% sodium dodecyl sulfate, 4% sucrose, and 0.02% bromophenol blue were added to stop the reactions. To disrupt any hydrogen bonding that might exist between the cohesive termini produced as the result of endo R·EcoRII restriction (4), the resulting solutions were incubated at 55 C for 5 min and then immediately placed at 0 C. Before analysis, the samples were incubated briefly at 37 C to dissolve the sodium dodecyl sulfate. The samples were layered on a 1.8% agarose (Marine Colloids, Inc.) slab gel containing 0.5 μg of ethidium bromide per ml. The gel was prepared in the holder described by B. Sugden, R. Roberts, J. Sambrook, and B. DeTroy (Anal. Biochem., in press) but modified in size (14 by 16 by 0.6 cm) to fit the electrophoresis apparatus described by Studier (26). The buffer in the gel and the running buffer consisted of: 40 mM Tris-acetate, pH 7.4, 20 mM sodium acetate, 2 mM EDTA, and 0.5 μg of ethidium bromide per ml. The gel was run at 80 V. The samples analyzed were: (a) 0.8 μg of simian virus 40 DNA; (b) 0.8 μg of simian virus 40 DNA digested with endo R·EcoRII; (c) 1.5 μg of mec⁺ f1 RFI; (d) 1.5 μg of mec⁺ f1 RFI exposed to endo R·EcoRII; (e) 1 μg of mec⁻ f1 RFI; (f) 1 μg of mec⁻ f1 RFI digested with endo R·EcoRII; (g) 1.5 μg of mec⁺ f1 RFI digested with endo R·HindII; (h) 1.5 μg of mec⁺ f1 RFI digested with endo R·HaeIII. Six of the nine f1 HaeIII fragments (Horiuchi et al., in press) are visible on this gel and their positions are indicated by the letters A through F on the right side of the figure. O indicates the origin. The Roman numerals on the left side of the figure indicate, from top to bottom, the positions of f1 RFII (a circular, double-stranded DNA molecule containing at least one single strand nick), RFI, and RFIII, respectively. The figure is a picture of a single slab gel taken with a red filter under UV illumination.*

this cleavage map of f1, several experiments were carried out. ^{32}P-labeled *mec*$^-$ f1 RFI was digested with both endo R·*Eco*RII and endo R·*Hae*III. After electrophoresis of the digest on a 4% polyacrylamide cylindrical gel, the gel was fractionated and the pieces were processed for scintillation counting (Horiuchi et al., in press). Figure 2 shows the results of such an experiment. Fragments HaeIII-B and HaeIII-C virtually disappeared. Three new fragments which migrate between HaeIII-A and the triplet band that contains HaeIII-D, HaeIII-E, and HaeIII-F (Horiuchi et al., in press) are observed. The percentage of the total ^{32}P counts per minute found in the five peaks of radioactivity (labeled HaeIII-A, HaeIII-D,E,F, HaeIII-G, HaeIII-H, and HaeIII-I in Fig. 2) indicate that the seven corresponding fragments (i.e., HaeIII-A and HaeIII-D through HaeIII-I) were probably all present intact and, hence, that these fragments do not contain an endo R·*Eco*RII cleavage site (data not shown). Thus, one of the two endo R·*Eco*RII cleavage sites is located within HaeIII-B and the other within HaeIII-C.

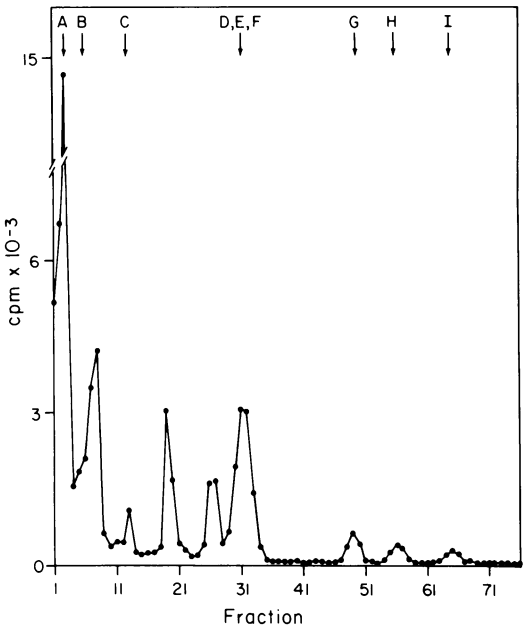

FIG. 2. *Electrophoresis of fragments produced by the digestion of mec*$^-$ *f1 RFI with endo R·EcoRII and endo R·HaeIII. ^{32}P-labeled mec*$^-$ *f1 RFI (1.9 μg; about 5.8 × 10^5 counts/min) was digested with endo R·EcoRII at 35 C for 16 h in a 100-μl reaction mixture containing 90 mM Tris-hydrochloride, pH 7.4, 5 mM $MgCl_2$, and 150 μg of Pentex bovine albumin (Miles Laboratories, Inc.) per ml. After removing protein by extraction with phenol, the DNA was ethanol precipitated, dissolved, and ethanol precipitated again. After digesting a second time with endo R·EcoRII as described above, 3/10 of the total was DNA digested with endo R·HaeIII at 37 C for 1 h. The reaction was stopped and prepared for analysis as described in the legend to Fig. 1. The DNA was analyzed on a 4% polyacrylamide cylindrical gel as previously described (Horiuchi et al., in press). The letters A through I indicate the positions of the nine f1 HaeIII fragments (Horiuchi et al., in press) as determined from a parallel gel.*

To determine the size of the fragments produced by the endo R·*Eco*RII cleavage of HaeIII-B and HaeIII-C, an endo R·*Hae*III digest of ^{32}P-labeled *mec*$^-$ f1 RFI was fractionated on an agarose slab gel. HaeIII-B and HaeIII-C were extracted from the gel and subjected to restriction by endo R·*Eco*RII. The results of this experiment are shown in Fig. 3. The sizes of the new fragments produced can be estimated on the 2.5 to 7.5% polyacrylamide gradient slab gel by comparing their mobility versus that of the f1 HaeIII fragments, the sizes of which have already been determined by Horiuchi et al. (in press). HaeIII-B, whose size is 26% of f1 RFI, is cut by endo R·*Eco*RII into a 19 and a 5.7% f1 RFI size fragment, whereas HaeIII-C, 13% of f1 RFI, is cut into a 8.8 and a 4.3% f1 RFI size fragment (Table 1). The 4.3% fragment is not detectable on the gel shown in Fig. 2 because it co-migrates with the triplet band which contains fragments HaeIII-D,E, and F (4.8, 4.6, and 4.6% of f1 RFI, respectively).

To order the fragments produced by the endo R·*Eco*RII digestion of HaeIII-B and HaeIII-C and, thus, locate the endo R·*Eco*RII cleavage sites on the f1 cleavage map, the results of the following additional experiment were needed. Endo R·*Hin*dII breaks f1 RFI at one specific location (Horiuchi et al., in press). Three fragments are produced by the combined action of endo R·*Eco*RII and endo R·*Hin*dII on *mec*$^-$ f1 RFI (data not shown). Comparison of the gel pattern obtained from such a digest with that obtained only if endo R·*Eco*RII is used (data not shown) reveals that: (i) the endo R·*Hin*dII cleavage site is located in the RII-A fragment; and (ii) the smaller fragment produced by the endo R·*Hin*dII cleavage of RII-A, i.e., RII-A2, is larger than RII-B.

Combining all of the results presented above, the location of the two endo R·*Eco*RII cleavage sites in relation to the endo R·*Hin*dII and endo R·*Hae*III cleavage sites must be the one indicated on the partial cleavage map shown in Fig. 4. First, since RII-B is smaller than RII-A2 (the smaller fragment produced by the action of endo R·*Hin*dII on RII-A; see above), the endo R·*Eco*RII site in HaeIII-B must be closer to the HaeIII-B/HaeIII-C boundary than to the HaeIII-B/HaeIII-D boundary. If the endo R·*Eco*-

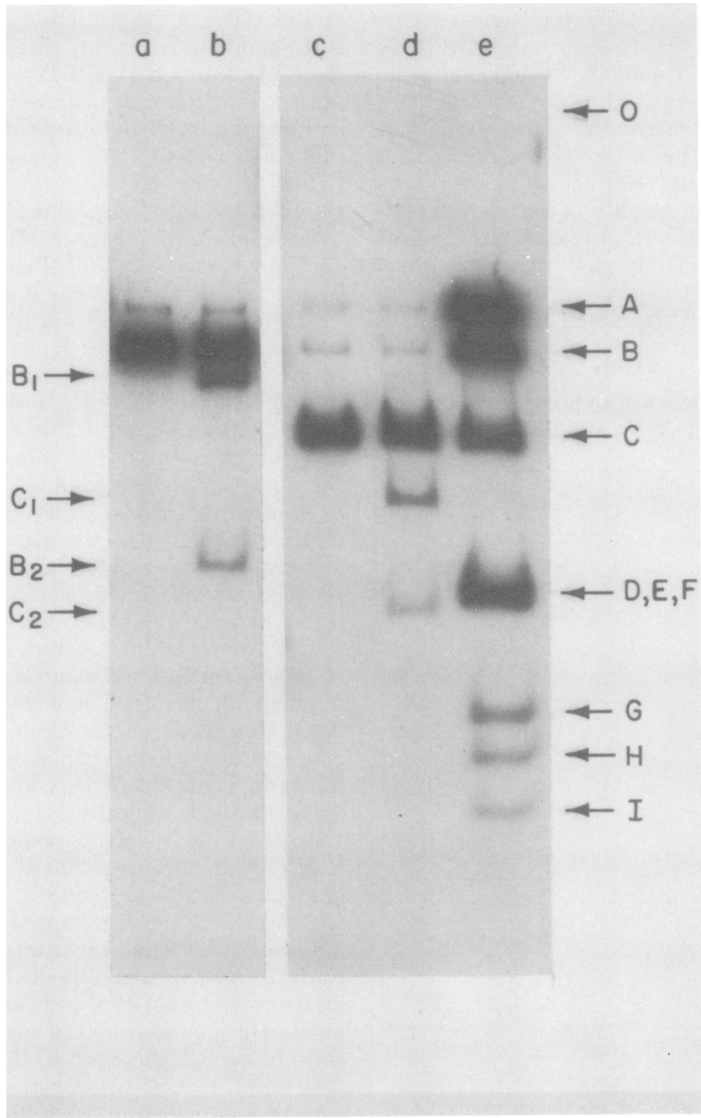

FIG. 3. *Digestion of f1 HaeIII-B and HaeIII-C fragments with endo R·EcoRII. Samples layered on the 2.5 to 7.5% polyacrylamide gradient slab gel (Horiuchi et al., in press) were: (a) HaeIII-B; (b) HaeIII-B digested with endo R·EcoRII; (c) HaeIII-C; (d) HaeIII-C digested with endo R·EcoRII; (e) f1 RFI digested with endo R·HaeIII. The positions of the nine f1 HaeIII fragments (Horiuchi et al., in press) are indicated on the right side of the figure by the letters A through I. O indicates the origin. The lettered arrows on the left side of the figure indicate the positions of the fragments produced by the endo R·EcoRII digestion of HaeIII-B (B_1 and B_2) and HaeIII-C (C_1 and C_2). The conditions for the endo R·HaeIII digestion of mec⁻ f1 RFI and the isolation of the HaeIII-B and the HaeIII-C fragments have been described (Horiuchi et al., in press). The conditions for the endo R·EcoRII digestion were as before (legend to Fig. 1). The endo R·EcoRII digestion of the isolated HaeIII fragments was not complete (b and d). Complete digestion has been achieved but only when larger amounts of DNA substrate are present (Vovis, unpublished data). This observation is currently under investigation. The figure is a picture of the radioautograph of a single slab gel.*

RII cleavage site were closer to the HaeIII-B/HaeIII-D boundary, then RII-B would have to be larger, not smaller, than RII-A2. Given the location of the endo R·*Eco*RII cleavage site in HaeIII-B, the other endo R·*Eco*RII site, which is in HaeIII-C, must be closer to the HaeIII-C/HaeIII-E boundary than to the HaeIII-C/HaeIII-B boundary. This follows because RII-B, although smaller than RII-A2, is larger than HaeIII-C (Fig. 1). If the endo R·*Eco*RII site

TABLE 1. *Size of fragments produced by endo R·EcoRII digestion of HaeIII-B and HaeIII-C*[a]

Substrate	Fragments produced	Size[b] (% of f1 RFI)
HaeIII-B		26
	1	19
	2	5.7
HaeIII-C		13
	1	8.8
	2	4.3

[a] Summary of the data from Fig. 3.

[b] The sizes of the fragments produced by the endo R·*Eco*RII cleavage of HaeIII-B and HaeIII-C were estimated by comparing the mobility of the products with that of the HaeIII fragments, the sizes of which have already been determined (Horiuchi et al., in press).

were closer to the HaeIII-C/HaeIII-B boundary, then RII-B would have to be smaller, not larger, than HaeIII-C.

The relevant distances, expressed as percentage of f1 RFI, are indicated on the partial cleavage map shown in Fig. 4. The estimated sizes for the two fragments produced by the endo R·*Eco*RII digestion of f1 are, thus, 14.5 and 85.5% of f1 RFI. The relative size of each RII fragment was also determined from electron microscopy examination. The ratio of the length of fragment RII-A to RII-B was found to be about 6.4 (G. Turgeon and G. F. Vovis, unpublished data).

Marker rescue experiments. To determine the genetic sites contained on fragments RII-A and RII-B, *mec*⁻, wild-type f1 RFI was digested with endo R·*Eco*RII and fractionated electrophoretically on agarose slab gels. After extraction and concentration, the isolated fragments were denatured with alkali, neutralized; and viral DNA containing a given amber mutation was added. After annealing, the partial duplex molecules were used to transfect *Su*⁻ *E. coli* made permeable to exogenous DNA by treatment with $CaCl_2$ (29). Production of wild-type phage and, hence, formation of a plaque on the *Su*⁻ bacterial lawn is expected if the DNA fragment covers the site of the amber mutation in the viral DNA strand (7, 13).

Horiuchi et al. (in press) have determined which genetic markers are rescued by fragments HaeIII-B and HaeIII-C, both of which contain an endo R·*Eco*RII cleavage site (Fig. 4). Therefore, the amber mutants rescued by HaeIII-B and HaeIII-C were the mutants used in the RII experiments. Table 2 shows the results of these experiments. Amber mutants R171 (gene III), R16 (gene V), and R119 (gene II) were rescued by fragment RII-A, whereas R4 (gene III), 8H1 (gene VIII), and 7H1 (gene VII) were rescued by RII-B. Thus, one endo R·*Eco*RII cleavage site is located within gene III between the polar R4 and the less polar R171 amber mutations, both of which are covered by fragment HaeIII-C (Horiuchi et al., in press). The location of the other endo R·*Eco*RII cleavage site, the one within HaeIII-B, can not be pinpointed by this experiment. However, the fact that a gene

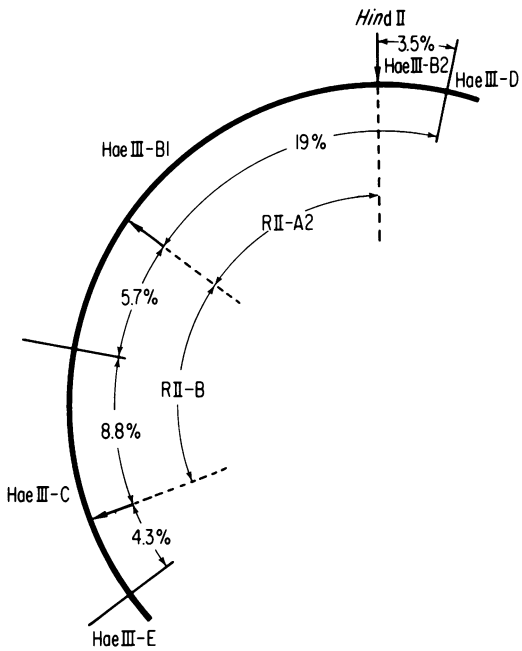

FIG. 4. *Location of the endo R·EcoRII cleavage sites on the f1 cleavage map. The relevant portion of the f1 cleavage map determined by Horiuchi et al. (in press) is depicted by the heavy curved line. HaeIII-D, HaeIII-B, HaeIII-C, and HaeIII-E are the four DNA fragments produced by the endo R·HaeIII digestion of f1 RFI that constitute this portion of the cleavage map. HaeIII-B is 26% of f1 RFI, HaeIII-C is 13% of f1 RFI (Horiuchi et al., in press). The arrow labeled HindII (the outer side of the heavy curved line) indicates the location of the single endo R·HindII cleavage site in f1. Endo R·HindII breaks HaeIII-B into HaeIII-B1 and HaeIII-B2 (22 and 3.5% of f1 RFI, respectively; Horiuchi et al., in press). The two arrows on the inner side of the heavy curved line indicate the location of the two endo R·EcoRII cleavage sites in f1 (see text). RII-B is the smaller fragment produced by the digestion of mec⁻ f1 RFI by endo R·EcoRII. RII-A2 is the smaller fragment produced from RII-A by endo R·HindII, where RII-A is the larger fragment produced by the digestion of mec⁻ f1 RFI by endo R·EcoRII. The percentages are the sizes of the indicated DNA fragments expressed as percentage of f1 RFI (Table 1 and Horiuchi et al., in press).*

TABLE 2. *Rescue of amber mutations by RII fragments*

Viral strand	RII fragment		
	A	B	None
R171 (III)[a]	752[b]		2
R4 (III)		175	4
8H1 (VIII)	6	110	10
7H1 (VII)	9	78	8
R16 (V)	236	4	6
R119 (II)	410		5
No viral DNA	6	0	0

[a] The Roman numeral in parenthesis refers to the gene in which the amber mutation is located.

[b] The number is the number of plaques obtained per plate from the $CaCl_2$-treated, *Su⁻ E. coli* cells that were transfected with amber mutant viral DNA to which denatured RII fragments were hybridized. Positive results are underlined for convenience.

TABLE 3. *Rescue of gene V and VII amber mutations by RII fragments*

Viral strand		RII fragment		
Gene	Amber	A	B	None
V	R13	169[a]	17	0
	R16	124	9	0
	R85	257	4	0
	R99	317	11	0
	R148	7	1,064	0
VII	7H1	6	304	0
	R84	7	544	0
	R100	9	1,115	0
	R147	9	~1,300	0
No viral DNA		5	0	0

[a] Number of plaques obtained per plate from the $CaCl_2$-treated, *Su⁻ E. coli* cells that were transfected with amber mutant viral DNA to which denatured RII fragments were hybridized. Positive results are underlined for convenience.

III, a gene VIII, and a gene VII amber mutant are rescued by RII-B whereas a gene V and a gene II amber mutant are rescued by RII-A allows the ordering of genes V and VII and, thus, establishes the gene order in this region as II, V, VII, VIII, III (see reference 15). Previously, from recombination studies, the gene order was suggested to be II, VII, V, although in vivo polarity was more consistent with the order being II, V, VII (Lyons, Ph.D. Thesis).

In an attempt to establish the location of the endo R·*Eco*RII cleavage site found in HaeIII-B, in relation to genes V and VII, a marker rescue experiment was carried out using viral DNA from all of the independently isolated gene V and gene VII amber mutants available in this laboratory. The results are shown in Table 3. All four gene VII amber mutants were rescued by RII-B. Four of the five gene V amber mutants were rescued by RII-A, whereas one was rescued by RII-B. Therefore, the endo R·*Eco*RII cleavage site contained in fragment HaeIII-B is within gene V.

Correlating these results with the partial cleavage map shown in Fig. 4 and the restriction mapping and transfection experiments of Horiuchi et al. (in press), one can deduce the f1 map shown in Fig. 5. The outer circle represents the restriction map and the middle circle the genetic map. The inner circle, which is divided into 10 equal parts with the single endo R·*Hin*dII cleavage site as the reference point, provides a convenient way of estimating distances on both the physical and genetic maps.

Mapping the SB_1 site. Bacteriophage f1 contains two SB sites, the genetic sites which confer upon DNA sensitivity to restriction and modification by *E. coli* B ($r_B^+m_B^+$). The wild-type allele is designated SB^+ and the mutant allele SB^0. The SB_2 site was localized by Horiuchi et al. (in press) within HaeIII-B2, the smaller fragment produced from HaeIII-B by the enzymatic action of endo R·*Hin*dII (see Fig. 4 or 5). The SB_1 site is within HaeIII-B1 (the larger fragment produced from HaeIII-B by endo R·*Hin*dII) and is tightly linked to gene V (15). Since an endo R·*Eco*RII cleavage site is located within gene V (Table 3), the following experiment was carried out to determine the location of the SB_1 site relative to this endo R·*Eco*RII cleavage site. RII fragments were isolated from wild-type $SB_1^+SB_2^+$ f1 RFI. RII-A was annealed to viral DNA from R119 (amber mutation in gene II, $SB_1^0SB_2^+$) and RII-B to viral DNA from 7H1 (amber mutation in gene VII, $SB_1^0SB_2^+$). Several of the plaques obtained from the transfection of *E. coli* K38 (*Su⁻*, $r_K^+m_K^+$) were picked and the plating efficiency of the am^+ phage therein on $r_B^+m_B^+$ versus $r_K^+m_K^+$ *E. coli* was determined. (It is important to remember that f1 is sensitive to B-specific restriction and modification but inert to the allelic [3] K-specific system [1].) The results (Table 4) clearly indicate that the SB_1 site is within RII-A. Sixty-seven percent of the plaques resulting from the rescue of the gene II amber mutant by RII-A were made up of phage that were also $SB_1^+SB_2^+$, i.e., the SB_1^0 allele of the viral DNA was replaced by an SB_1^+ allele which must have been present on the RII-A fragment. All of the examined plaques resulting from the rescue of the gene VII amber mutation by RII-B were made up of phage that were still like the input viral DNA, i.e., $SB_1^0SB_2^+$.

RII-A and RII-B directed in vitro protein

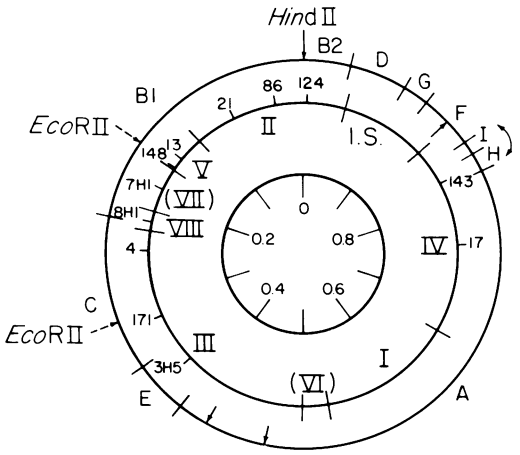

FIG. 5. *Physical and genetic map of bacteriophage f1. The outer circle, representing the physical map, shows the location of the endo R·HaeIII fragments (A, B, . . ., I), the cleavage sites of endo R·EcoRII (the two dashed arrows labeled EcoRII on the outside of the circle) and endo R·HindII (the solid arrow labeled HindII on the outside of the circle), and the three cleavage sites of endo R·HaeII (the solid arrows on the inside of the circle). The location of the endo R·EcoRII cleavage sites was determined as described in the text. The cleavage sites of the other enzymes were located before (Horiuchi et al., in press). The previously ambiguous order of fragments HaeIII-D and HaeIII-G has since been determined by cleavage mapping with endo R·HpaII to be as shown (Horiuchi, unpublished data). The order of fragments HaeIII-H and HaeIII-I is still ambiguous and is so indicated in the figure. However, Schoenmakers (personal communication) has recently demonstrated that the order of the two corresponding fragments of the related bacteriophage M13 is as shown in the figure. The middle circle represents the genetic map. The order of the genes (Roman numerals) was determined by Lyons and Zinder (15) except for genes V and VII, the order of which was determined in the present study (Tables 2 and 3). The sizes of the genes were estimated from the molecular weight of the in vitro gene products (17) with the exception of genes VI and VII, the Roman numerals of which are shown in parenthesis because the gene products have not been identified and, hence, the gene sizes are unknown. The locations of the mutant sites within the genes, shown on the outside of the middle circle, were determined for R124, R86, R21, R13, R17, and R143 from the sizes of the amber fragments synthesized in vitro (17; Model and Enea, unpublished data). The other mutant sites shown were localized on the restriction fragments by transfection experiments (Tables 2 and 3; Horiuchi et al., in press). Direction of transcription and translation is counterclockwise (17). The physical and genetic maps were correlated to each other by: (i) estimating that the total f1 genome corresponds to 250,000 daltons of protein (or about 6,800 nucleotide base pairs); (ii) assuming that the endo R·HaeIII cleavage site between HaeIII-B and HaeIII-C is located within gene VIII; and (iii) determining that the size of the in vitro amber fragment of R143 is about 40,000 daltons (Model and Enea, unpublished data). Given these estimations, the relationship between the physical and genetic maps should be as shown in the figure. From such a correlation, the existence of an intergenic space (I.S.) of considerable size between genes IV and II is indicated and is so shown in the figure. The I.S. contains the initiation site for replication of the f1 viral strand (Horiuchi, unpublished data). The inner circle is divided into 10 equal map units with the endo R·HindII cleavage site as the reference point.*

synthesis. The results of the marker rescue experiments described above and the order of the genes in the genetic map of f1 (see Fig. 5) indicate that the genetic content of the RII fragments must be: (i) RII-A: part of gene III, genes VI, I, IV, II, and part of gene V; and (ii) RII-B: part of gene V, genes VII, VIII, and part of gene III. The protein products have been identified for all f1 genes except VI and VII (14, 17). Thus, it was feasible to ask whether the appropriate f1 proteins are synthesized in vitro under the direction of each RII DNA fragment.

The isolated RII fragments were added to the coupled transcription-translation system described by Model and Zinder (17) and the polypeptides synthesized were fractionated on an exponential polyacrylamide gradient gel. The results of such an experiment are shown in Fig. 6. Short radioautographic exposure of the acrylamide gels shows that substantial amounts of only one f1-specific polypeptide appear to be synthesized in each RII-directed reaction. With RII-B, a polypeptide that co-migrates with the major coat protein, the product of gene VIII (16), is synthesized (compare 3 and 4 in Fig. 6). No such protein is present in the RII-A-directed reaction (1 in Fig. 6). With RII-A, the f1 polypeptide synthesized comigrates with the χ protein (compare 1 and 4 in Fig. 6). No such protein was present in the RII-B-directed reaction (3 in Fig. 6).

The χ protein was so named because, although apparently f1 specified, it has not yet been assigned to a f1 gene (14, 17). However, since a HaeIII-B-directed reaction also synthesizes the χ protein (Model and Horiuchi, unpublished data), the region of the genome responsible for its synthesis is defined by the 19% of f1 RFI DNA fragment produced from HaeIII-B by endo R·EcoRII (see Fig. 4 and 5).

Radioautographs exposed for a longer time (e.g., 1 month instead of 135 h) suggest the presence of other f1-specific polypeptides in the RII-A-directed reaction (data not shown), i.e., the products of genes IV and II. Stimulation of protein synthesis by both RII-A and RII-B is poor, however, and the amounts of gene IV and gene II products synthesized are not suffi-

TABLE 4. *Determination of which RII fragment contains the SB_1 site*[a]

RII fragment	Viral strand	Number of am^+ phage per plate	Number of SB^+ sites in am^+ phage
A	R119 ($II_{86}SB_1{}^0SB_2{}^+$)	782	SB=2 (8/12) SB=1 (4/12)
A	None	18	
B	7Hl ($VII_{7Hl}SB_1{}^0SB_2{}^+$)	218	SB=1 (12/12)
B	None	0	
None	R119 ($II_{86}SB_1{}^0SB_2{}^+$)	6	SB=1 (4/4)
None	7H1 ($VII_{7H1}SB_1{}^0SB_2{}^+$)	2	SB=1 (2/2)

[a] $CaCl_2$-treated cells of K38($Su^-, r_K{}^+m_K{}^+$) were transfected with $SB_1{}^0SB_2{}^+$, amber mutant viral DNA to which denatured RII fragments from wild-type RF($SB_1{}^+SB_2{}^+$) were hybridized. Several of the am^+ plaques, the exact number is indicated, were picked and the plating efficiency (eop) of the phage therein was determined on K336B($r_B{}^+m_B{}^+$) relative to K38($r_K{}^+m_K{}^+$). SB=2 refers to $SB_1{}^+SB_2{}^+$ phage (eop=0.001), whereas SB=1, in this case, refers to $SB_1{}^0SB_2{}^+$ phage (eop=0.03).

ciently above the background levels (synthesis without added DNA) to make these results significant. Nevertheless, the absence of the gene VIII product from the RII-A-directed reaction can be considered significant since the major coat protein is a major product both in vivo and in vitro (11, 14, 16, 17) and its synthesis is observed when RII-B is used as template.

The presence of the gene VIII protein in the RII-B-directed reaction indicates the existence of an RNA promoter in the region contained within RII-B, i.e., from within gene V to the beginning of gene VIII (the order of transcription and translation on the genetic map is counterclockwise [Fig. 5], i.e., V, VII, VIII, III [17]). The placement of an RNA promotor in this region of the genome will be discussed later in the context of some in vitro transcription-translation studies. The fact that fragment RII-B directs the synthesis of a polypeptide, which is apparently gene VIII (Fig. 6), correlates very well with the genetic data which indicates that fragment RII-B contains gene VIII (Table 2; see also Fig. 5).

DISCUSSION

We have demonstrated that f1 RFI isolated from an *E. coli* strain deficient in cytosine methylase activity is sensitive in vitro to restriction by endo R·*Eco*RII (Fig. 1). This result confirms a similar observation by Schlagman et al. (submitted for publication) who used DNA from the closely related bacteriophage fd (16). That the cleavage is due to endo R·*Eco*RII rather than some contaminating enzyme is consistent with the fact that the f1 fragments possess cohesive termini (G. Turgeon, G. F. Vovis, and C. Yehle, unpublished data) as do authentic RII fragments (4). Thus, as has already been pointed out (10, 22; Schlagman et al., submitted for publication), the cytosine methylase specified by the *mec* chromosomal locus apparently methylates the same DNA sequence recognized by endo R·*Eco*RII (4). However, confirmation must await DNA sequence studies.

f1 contains two endo R·*Eco*RII cleavage sites (Fig. 1-3). Given that the endo R·*Eco*RII digests of f1 and fd *mec*$^-$ RFI are indistinguishable from each other on a 1.8% agarose slab gel (Vovis and Schlagman, unpublished data), a good correlation exists between the number of

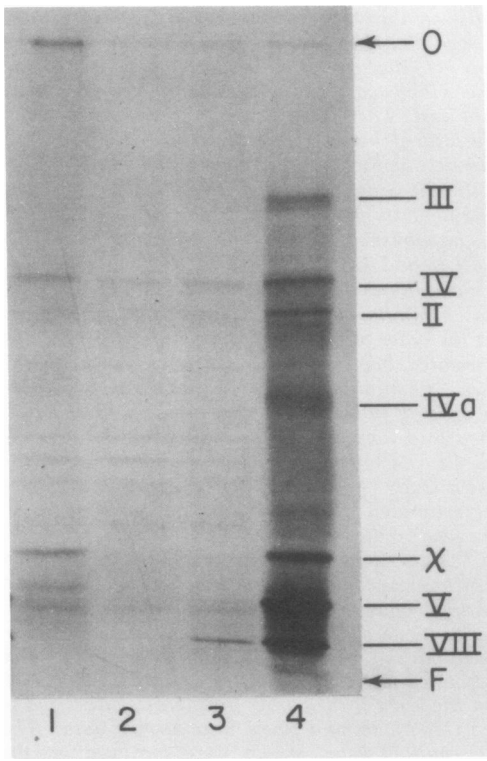

FIG. 6. *RII-A and RII-B directed in vitro protein synthesis. The following DNA samples were added to the coupled transcription-translation system described by Model and Zinder (17): (1) fragment RII-A; (2) no DNA; (3) fragment RII-B; (4) f1 RFI. The Roman numerals are gene identifications; χ is a f1-specific protein which has not yet been assigned to a gene (14, 17). O is the origin, F the front. The figure is a picture of the radioautograph of a single slab gel.*

5-methylcytosine residues found in fd viral DNA of phage grown in an N-3 plasmid containing *E. coli* strain that is *mec*$^+$ or *mec*$^-$ (between two to three; 9, 10, 22) and the number of endo R·*Eco*RII cleavage sites in f1 (two; Fig. 1-3).

The sizes of the two DNA fragments produced by the endo R·*Eco*RII digestion of *mec*$^-$ f1 RFI, RII-A and RII-B, are about 14.5 and 85.5% of f1 RFI, respectively (Table 1 and Fig. 4). These fragments have been localized on the f1 cleavage map of Horiuchi et al. (in press) (Fig. 4 and 5). Marker rescue experiments have determined the genetic content of each fragment and show that one of the two endo R·*Eco*RII breaks occurs within gene III and the other within gene V (Tables 2 and 3; see also Fig. 5). Furthermore, the marker rescue experiments have demonstrated not only that the RII-A fragment contains both SB sites (Table 4; Horiuchi et al., in press) but also, and more importantly, that the V-VII gene order must be II, V, VII, VIII (Table 2). During the course of this work, a similar conclusion regarding the V-VII gene order was also reached by van den Hondel, Weijers, Konings, and Schoenmakers (Eur. J. Biochem., in press) as the result of restriction mapping of M13 with endo R·*Hap*II (van den Hondel and Schoenmakers, in press).

The addition of isolated RII-B fragments to a coupled transcription-translation system results in the synthesis of the major coat protein (Fig. 6), the product of gene VIII (16). Thus, an RNA promoter for gene VIII must exist within that area of the genome defined by the RII-B DNA fragment (see Fig. 5). In vitro transcription studies with f1, fd, and M13 RFI and various restriction fragments of RFI (27; Seeburg and Schaller, J. Mol. Biol., in press; Chan, Model, and Zinder, submitted for publication) indicate the existence of an RNA promotor within the gene V-gene VII region of the genome. From this promotor, a pppG-initiated mRNA of approximately 10*S* is synthesized (27; Chan et al., submitted for publication). Only the gene VIII protein can be detected as being synthesized from such an RNA template (Chan et al., submitted for publication). Thus, the results of the RII-B directed protein synthesis experiment (Fig. 6) confirm these in vitro transcription and transcription-translation observations.

Implications for hybrid DNA molecule formation. The use of the cohesive termini produced by restriction enzymes to construct hybrid DNA molecules has already begun to be exploited. Prokaryotic and eukaryotic chromosomal and prokaryotic plasmid DNAs have been inserted into the *E. coli* pSC101 plasmid by such means (5, 6, 18). Similar experiments are also possible with phage DNA (19). Replication and packaging of hybrid DNA molecules into phage particles, however, require the presence of a helper phage if a required phage function is deleted. Nevertheless, the ease with which phage can be purified (31) makes this the method of choice for the amplification of hybrid DNA molecules. A more severe problem is that most phage particles are of a fixed size and, hence, only DNA of a limited size range can be packaged. This limitation greatly restricts the size of hybrid DNA molecules that are possible. Filamentous phage do not appear to suffer from such a disadvantage since the size of the phage particle may very well be determined by the size of the DNA molecule. Thus, the discovery of conditions for making filamentous phage DNA sensitive to endo R·*Eco*RII, a restriction enzyme which produces cohesive termini (4), allows the advantages offered by these phage to be exploited. Experiments exploiting these advantages are currently in progress.

ACKNOWLEDGMENTS

We wish to acknowledge S. Hattman for calling our attention to his work and that of his colleagues concerning the possible sensitivity of DNA from filamentous bacteriophage to restriction in vitro by endo R·*Eco*RII and then for communicating the results of experiments before publication. We are most thankful to P. Model for his invaluable assistance and for critically reading this manuscript. We are deeply indebted to Gillian Turgeon for isolating the viral DNAs and for carrying out many of the transfection experiments and to Carolyn McGill for doing the in vitro protein synthesis and then running the subsequent polyacrylamide gels.

This work was supported in part by grants from the National Science Foundation.

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