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Site-specific cleavage of single-stranded DNA by a *Hemophilus* restriction endonuclease

(f1 phage DNA/endo R·*Hae*III/fragments of single strands)

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ABSTRACT Single-stranded viral DNA of bacteriophage f1 is cleaved into specific fragments by endo R·*Hae*III, a restriction endonuclease isolated from *Hemophilus aegyptius*. The sites of the single strand cleavage correspond to those of the double strand cleavage. A single-stranded DNA fragment containing only one *Hae*III site is also cleaved by this enzyme. This observation suggests that the reaction of single-stranded DNA cleavage does not require the formation of a symmetrical double-stranded structure that would result from the intramolecular base-pairing between two different *Hae*III sites. Other restriction endonucleases may also cleave single-stranded DNA.

Various restriction endonucleases have been isolated which recognize specific base sequences in double-stranded DNA (1–3). Those that cleave DNA at specific sites (4–12) have been particularly useful for the physical dissection and subsequent analyses of DNA genomes. The recognition sites for these enzymes have been shown to have a 2-fold rotational symmetry (13–19) and, hence, the enzymes are believed to act only on double-stranded DNA.

Bacteriophage f1 contains a covalently closed, circular single-stranded DNA of 1.8×10^6 daltons (20). Upon infection of sensitive cells of *Escherichia coli*, replication of the phage DNA takes place through the formation of double-stranded, circular replicative form DNA (RF). The RF, consisting of viral and complementary strands, can be isolated from the f1-infected cells and used as a substrate for endonucleolytic reactions.

Endo R·*Hae*III*, a restriction endonuclease isolated from *Hemophilus aegyptius* (6), has been shown to cleave the RF of bacteriophage f1 to produce nine specific fragments which have been ordered to construct a circular map of the f1 genome (12). The present paper reports that endo R·*Hae*III cleaves single-stranded viral DNA of f1 at specific sites and that the sites of the single strand cleavage apparently correspond to those of the double strand cleavage.

MATERIALS AND METHODS

DNA. Single-stranded viral DNA of phage f1 was prepared as described previously (21). Covalently closed, circular replicative form DNA (RFI) of f1 was prepared by the modified procedure (12) of the method described by Model and Zinder (22). K415 (F^+ mec^- endo I^- thi^- $r_K^- m_K^+$), the *E. coli* strain used for the preparation of RFI, was very generously provided by Dr. S. Hattman (23). Preparation of ^{32}P-labeled DNA has been described (12).

Endonucleases. Endonucleases R·*Hae*III (6, 12), R·*Hae*II (11, 12), and R·*Hind* (4) were isolated as described previously (12). Endo R·*Eco*RII, isolated by the method described by Yoshimori (5), was supplied by Dr. G. F. Vovis.

Denaturation and Renaturation. Alkali denaturation and subsequent renaturation of DNA were carried out as described previously (24) except that acetic acid was used instead of NaH_2PO_4 to neutralize the alkali.

Gel Electrophoresis. Procedures for slab gel electrophoresis, radioautography, and extraction of DNA from the gel have been described previously (12). A gel containing a 2.5–7.5% gradient of polyacrylamide (25) was used for the analysis of DNA fragments, while a 1.4% agarose gel was used for the preparation of DNA fragments.

RESULTS AND DISCUSSION

Endo R·*Hae*III, a restriction endonuclease isolated from *H. aegyptius* (6), cleaves the double-stranded replicative form DNA (RF) of bacteriophage f1 at nine specific sites to yield nine fragments, which have been named *Hae*III-A through I. These fragments have been ordered so as to construct a circular cleavage map of the f1 genome (12) (See Fig. 1). Upon polyacrylamide gel electrophoresis, the endo R·*Hae*III digest of f1 RFI gives the pattern shown in Fig. 2a.

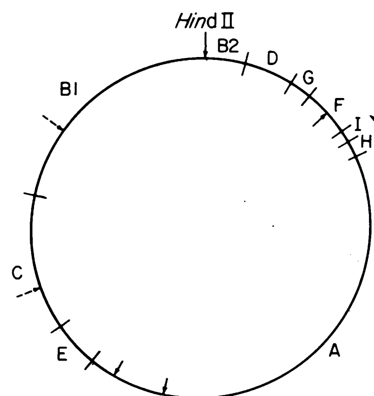


FIG. 1. Cleavage map of bacteriophage f1, which shows the location of the endo R·*Hae*III fragments (A,B,......,I), the cleavage sites of endo R·*Hin*dII (the solid arrow labeled *Hin*dII on the outside of the circle), endo R·*Hae*II (the three solid arrows on the inside of the circle), and endo R·*Eco*RII (the two dashed arrows on the outside of the circle) (ref. 12; Vovis, Horiuchi, and Zinder, submitted for publication).

Abbreviations: RF, replicative form DNA; RFI, a covalently closed, circular double-stranded DNA molecule.
*The nomenclature proposed by Smith and Nathans (32) for restriction-modification enzymes has been used, i.e., endo refers to endonuclease, R to restriction, *Eco* to *E. coli*, *Hin* to *H. influenzae*, *Hae* to *H. aegyptius*, *Hpa* to *H. parainfluenzae*, etc.

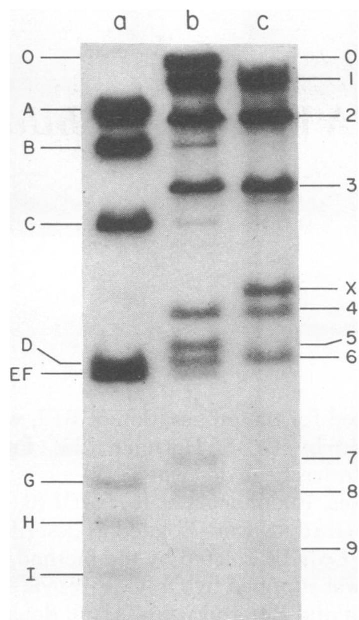


FIG. 2. Gel electrophoresis of phage f1 RFI and single-stranded DNA digested with endo R·*Hae*III. To prepare the samples (a) and (b), we incubated 1.4 μg of ^{32}P-labeled f1 RFI (34,000 cpm) with 4 μl of an endo R·*Hae*III preparation at 37° for 2 hr in an 80 μl reaction mixture containing 7 mM Tris·HCl, pH 7.4, and 7 mM $MgCl_2$. (a) A 20 μl aliquot of this reaction mixture was analyzed by gel electrophoresis as described previously (12) on a slab containing a 2.5–7.5% linear gradient of polyacrylamide. (b) The other aliquot (60 μl) was mixed with 19 μg of nonradioactive, intact, single-stranded viral DNA of f1, and was subjected to alkali denaturation, subsequent renaturation, and ethanol precipitation. The precipitate was dissolved in 20 μl of the reaction buffer described above and analyzed by gel electrophoresis. (c) Two and two-tenths micrograms of ^{32}P-labeled single-stranded f1 DNA (8400 cpm) was incubated with 10 μl of the endo R·*Hae*III preparation at 37° for 2 hr in the reaction buffer described above, and analyzed by gel electrophoresis. The figure is a radioautograph of a single slab gel. Double-stranded *Hae*III fragments (a) are indicated by capital letters on the left side of the figure. *Hae*III fragments of the viral strand (b) are indicated by Arabic numerals on the right side of the figure. For a description of fragment X, see *text*. O represents the origin. The intact molecules of both f1 RFI and single-stranded f1 DNA remain at the origin of the gel (data not shown).

In order to test whether this endonuclease can cleave f1 single-stranded DNA, we incubated the viral DNA extracted from purified ^{32}P-labeled f1 phage with the enzyme and then analyzed it by gel electrophoresis. The result shown in Fig. 2c reveals the production of eight discrete fragments. This in itself indicates that the endo R·*Hae*III preparation cleaved the single-stranded f1 DNA at specific sites.

To examine whether the sites of the single-stranded DNA cleavage correspond to the sites of the RF cleavage, we carried out the following experiment. ^{32}P-Labeled f1 RFI was incubated in the presence of endo R·*Hae*III. After the reaction was stopped, a 40-fold molar excess of nonradioactive, single-stranded viral f1 DNA was added, and the mixture was subjected to alkali denaturation and subsequent renaturation. By this procedure most of the complementary strand component of the *Hae*III fragments was annealed to the full-length viral strand. The hybrid molecules thus formed remain at the top of the gel upon electrophoresis, leaving the viral strand component of the fragments free to migrate into the gel. Gel electrophoretic analysis of this sample thus allows one to observe only the viral strand component of the *Hae*III fragments. The result shown in Fig. 2b reveals nine single-stranded fragments, the positions of which are marked on the figure by Arabic numerals 1–9. An additional top band is due to the radioactivity of a portion of the complementary strand hybridized to the full-length viral strand. Additional faint bands due to the self-annealing of a small portion of the *Hae*III fragments are also seen in the figure. The striking resemblance between the pattern of the viral strand from the double strand digest (Fig. 2b) and the pattern of the viral strand digest (Fig. 2c) is obvious: among the eight bands shown in Fig. 2c seven had a mobility identical to that of the bands shown in Fig. 2b. This strongly suggests that the sites of the single strand cleavage correspond to those of the double strand cleavage. Apparently one of the nine cleavage sites was not cleaved when single-stranded DNA was used as the substrate, yielding a fragment that is labeled X in Fig. 2. By use of a higher concentration of endo R·*Hae*III and a longer incubation time, we have observed conversion of a portion of fragment X into fragments 5 and 7 (data not shown). Therefore, the resistance of this site in the single-stranded form to the enzyme is quantitative rather than qualitative. Quantitative differences in the sensitivity among other *Hae*III sites to the single strand cleavage have also been observed by using lower enzyme concentrations (data not shown). The results described above strongly suggest that the cleavage of the single-stranded DNA is catalyzed by endo R·*Hae*III, not by a contaminating enzyme in the preparation.

In order to correlate the viral strand fragments (Fig. 2c) to the double-stranded fragments (Fig. 2a), we cut out portions of the dried gel that correspond to each radioautographic band and measured the radioactivity. The result shown in Table 1 indicates that the viral strand fragments 1, 2, 3, 7, 8, and 9 (see Fig. 2c) correspond to the double-stranded fragments A, B, C, G, H, and I (see Fig. 2a), respectively. Preliminary results of experiments in which RF fragments pro-

Table 1. Radioactivity found in the area of a slab gel corresponding to the radioautographic bands from the endo R·*Hae*III digests of phage f1 RFI and single-stranded DNA[a]

(a) Double-stranded fragments of RFI digest		(b) Viral strand fragments of RFI digest		(c) Viral strand fragments of viral DNA digest	
Band[b]	Radioactivity (%)	Band[b]	Radioactivity (%)	Band[b]	Radioactivity (%)
A	42	1	37	1	40
B	26	2	27	2	26
C	13	3	14	3	14
DEF	14	4	6	4	5
		5	6		
		6	4	6	5
G	2.6	7	2.5		
H	1.7	8	(2.5)[c]	8	1.9
I	1.3	9	1.0	9	1.2
				X	6.5

[a] A slab gel was prepared in the same way as that shown in Fig. 2. The area of the gel corresponding to each radioautographic band was cut out and the radioactivity was measured in a liquid scintillation counter.

[b] For the nomenclature of the bands, see Fig. 2.

[c] Includes the radioactivity due to self-annealing of the fragment *Hae*III-G.

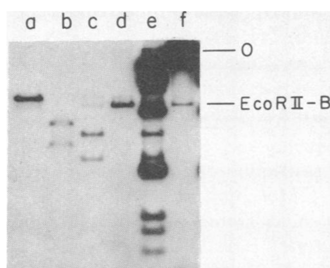
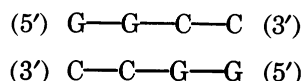


FIG. 3. Cleavage of the viral strand of fragment *Eco*RII-B by endo R·*Hae*III. ^{32}P-Labeled and ^{3}H-labeled preparations of *Eco*-RII-B were prepared by incubation of radioactive f1 RFI preparations with endo R·*Eco*RII and subsequent agarose gel electrophoresis as described elsewhere (Vovis, Horiuchi, and Zinder, submitted for publication). The viral strand of *Eco*RII-B was isolated as follows. Seventeen-hundredth pmol of ^{32}P-labeled *Eco*RII-B (6000 cpm) was mixed with 17 pmol of nonradioactive, intact single-stranded viral DNA of f1, subjected to denaturation and renaturation, concentrated by ethanol precipitation, and centrifuged in a 5–20% neutral sucrose gradient (24) (45,000 rpm for 3 hr at 18° in a Spinco SW50.1 rotor). Cerenkov counting of the collected fractions showed that approximately half the radioactivity layered had moved about three-tenths the length of the gradient, while the other half moved about three-quarters the length of the gradient. The slower sedimenting radioactive fractions were combined, dialyzed, and precipitated in ethanol in the presence of 1 μg of lambda phage DNA as carrier. The precipitate of the viral strand of *Eco*RII-B thus obtained (0.15 pmol) was dissolved in 40 μl of reaction buffer (7 mM Tris·HCl, pH 7.4, 7 mM $MgCl_2$). (a) A 10 μl aliquot of the viral strand of *Eco*RII-B was electrophoresed on the gel. (b) Another 10 μl aliquot was run after treatment with 7.5 μl of endo R·*Hae*III at 37° for 2 hr. The remaining 20 μl of ^{32}P-labeled viral strand of *Eco*RII-B was mixed with 3.0 pmol of ^{3}H-labeled double-stranded *Eco*RII-B, subjected to denaturation and renaturation, and precipitated in ethanol. The precipitate was dissolved in 20 μl of the reaction buffer. (c) A 10 μl aliquot of the renatured *Eco*RII-B thus obtained was treated with 7.5 μl of endo R·*Hae*III, and then run on the gel. (d) The other 10 μl of the renatured *Eco*-RII-B was run directly. (e) ^{32}P-labeled RFI treated with a mixture of endo R·*Hae*III and endo R·*Eco*RII. (f) ^{32}P-labeled RFI treated with endo R·*Eco*RII. The positions of the origin (O) and double-stranded *Eco*RII-B are indicated by arrows. Conditions for the gel electrophoresis were identical to those in Fig. 2. The figure is a radioautograph of a single slab gel.

duced by endo R·*Hae*II plus endo R·*Hind* (see Fig. 1) were digested with endo R·*Hae*III and subsequently subjected to denaturation and renaturation in the presence of an excess amount of intact viral strand have indicated that the single-stranded fragments 1, 2, 3, 4, 5, 6, 7, 8, and 9 are viral strands of *Hae*III fragments A, B, C, D, F, E, G, H, and I, respectively (data not shown).

Parenthetically it should be noted that in most gel systems double-stranded fragments of similar size are difficult to resolve (see fragments D, E, and F in Fig. 2a). However, the gel electrophoretic separation of their single-stranded counterparts can be much better (see fragments 4, 5, and 6 in Fig. 2b), probably because the mobility of single-stranded molecules reflects structure and base composition in addition to size.

Many restriction endonucleases have been shown to cleave double-stranded DNA within specific base sequences that have a 2-fold rotational symmetry (14–19). For instance, endo R·*Hae*III, the enzyme used in the present study, cleaves double-stranded DNA within the following base sequence



(K. Murray, cited in ref. 26). Since there are nine *Hae*III-sensitive sites on the f1 RF (12), it might be that the enzyme cleaves single-stranded f1 DNA only when two different *Hae*III sites on the molecule pair, even transiently, to form a double-stranded symmetrical structure. Alternatively, the enzyme may act directly on the single-stranded structure as a base-sequence-specific endonuclease like T4 endonuclease IV (27–30). To distinguish between these possibilities, we tested a single-stranded DNA fragment that contains only one *Hae*III site for its sensitivity to endo R·*Hae*III. Endo R·*Eco*RII cleaves f1 and fd RFI at two sites, producing two fragments, *Eco*RII-A and *Eco*RII-B (Vovis, Horiuchi, and Zinder, submitted for publication; S. Schlagman, S. Hattman, M. S. May, and L. Berger, submitted for publication). The fragment *Eco*RII-B contains only one endo R·*Hae*III site, the site between *Hae*III-B and *Hae*III-C (see Fig. 1). We, therefore, cleaved ^{32}P-labeled f1 RFI with endo R·*Eco*-RII, and isolated fragment *Eco*RII-B by gel electrophoresis in a 1.4% agarose slab. The viral strand of *Eco*RII-B was isolated by denaturation and renaturation in the presence of an excess of full-length viral DNA followed by subsequent neutral sucrose gradient centrifugation. When the viral strand of *Eco*RII-B thus obtained was incubated in the presence of endo R·*Hae*III, it was cleaved into two fragments as shown in Fig. 3a versus b. In addition, if the isolated viral strand of *Eco*RII-B is hybridized to an excess of alkali-denatured, nonradioactive *Eco*RII-B, it shows on gel electrophoresis the mobility of authentic *Eco*RII-B, and gives, upon cleavage with endo R·*Hae*III, two fragments which co-migrate with the double-stranded fragments obtained by the cleavage of RFI with a mixture of endo R·*Hae*III and endo R·*Eco*RII (Fig. 3c–f). These results indicate that endo R·*Hae*III can cleave a single-stranded DNA molecule that contains only one *Hae*III site. We cannot completely exclude the possibility that fragment *Eco*RII-B contains a second *Hae*III site that is located close to the first one, although such a hypothetical short *Hae*III fragment has not been detected. The simplest interpretation of our results is that intramolecular pairing between two *Hae*III sites is not required for the cleavage of single-stranded DNA. The relationship betwen recognition mechanisms of double-stranded and single-stranded DNAs by this enzyme remains to be further elucidated.

One of the obvious questions of interest is whether the cleavage of single-stranded DNA is a general characteristic of restriction endonucleases. Endo R·*Eco*B, which cleaves DNA at sites different from the DNA recognition sites (24), is inactive on single-stranded DNA (21, 31). Among the restriction endonucleases that produce site-specific cleavages, endo R·*Hind* appears not to cleave alkali-denatured T7 DNA (4). However, our preparation of endo R·*Hpa*II isolated from *H. parainfluenzae* (7, 8) produces discrete fragments with f1 viral DNA as the substrate (data not shown), although, in this instance, we have not yet tested whether the cleavage is due to endo R·*Hpa*II itself or another contaminating enzyme. It, therefore, appears rather likely that the cleavage of single-stranded DNA is not peculiar to endo R·*Hae*III.

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1. Arber, W. (1974) "DNA modification and restriction," *Progr. Nucl. Acid Res. Mol. Biol.* **14,** 1–37.
2. Meselson, M., Yuan, R. & Heywood, J. (1972) "Restriction and modification of DNA," *Annu. Rev. Biochem.* **41,** 447–466.
3. Boyer, H. W. (1971) "DNA restriction and modification mechanisms in bacteria," *Annu. Rev. Microbiol.* **25,** 153–176.
4. Smith, H. O. & Wilcox, K. W. (1970) "A restriction enzyme from *Hemophilus influenzae,* I. Purification and general properties," *J. Mol. Biol.* **51,** 379–391.
5. Yoshimori, R. N. (1971) "A genetic and biochemical analysis of the restriction and modification of DNA by resistance transfer factors," Ph.D. Dissertation, University of California, San Francisco.
6. Middleton, J. H., Edgell, M. H. & Hutchison, C. A., III (1972) "Specific fragments of ϕx174 deoxyribonucleic acid produced by a restriction enzyme from *Haemophilus aegyptius,* endonuclease Z," *J. Virol.* **10,** 42–50.
7. Gromkova, R. & Goodgal, S. H. (1972) "Action of *Haemophilus* endonuclease on biologically active deoxyribonucleic acid," *J. Bacteriol.* **109,** 987–992.
8. Sharp, P. A., Sugden, B. & Sambrook, J. (1973) "Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose-ethidium bromide electrophoresis," *Biochemistry* **12,** 3055–3063.
9. Takanami, M. & Kojo, H. (1973) "Cleavage site specificity of an endonuclease prepared from *Haemophilus influenzae* strain H-I," *FEBS Lett.* **29,** 267–270.
10. Takanami, M. (1973) "Specific cleavage of coliphage fd DNA by five different restriction endonucleases from *Haemophilus* genus," *FEBS Lett.* **34,** 318–322.
11. Roberts, R. J., Breitmeyer, J. B., Tabachnik, N. F. & Myers, P. A. (1975) "A second specific endonuclease from *Haemophilus aegyptius,*" *J. Mol. Biol.* **91,** 121–123.
12. Horiuchi, K., Vovis, G. F., Enea, V. & Zinder, N. D. (1975) "Cleavage map of bacteriophage f1: Location of the *Escherichia coli* B-specific modification sites," *J. Mol. Biol.*, in press.
13. Arber, W. & Linn, S. (1969) "DNA modification and restriction," *Annu. Rev. Biochem.* **38,** 467–500.
14. Kelly, T. J., Jr. & Smith, H. O. (1970) "A restriction enzyme from *Hemophilus influenzae,* II. Base sequence of the recognition site," *J. Mol. Biol.* **51,** 393–409.
15. Hedgpeth, J., Goodman, H. M. & Boyer, H. W. (1972) "DNA nucleotide sequence restricted by the RI endonuclease," *Proc. Nat. Acad. Sci. USA* **69,** 3448–3452.
16. Bigger, C. H., Murray, K. & Murray, N. E. (1973) "Recognition sequence of a restriction enzyme," *Nature New Biol.* **244,** 7–10.
17. Boyer, H. W., Chow, L. T., Dugaiczyk, A., Hedgpeth, J. & Goodman, H. M. (1973) "DNA substrate site for the *Eco*RII restriction endonuclease and modification methylase," *Nature New Biol.* **244,** 40–43.
18. Sugisaki, H. & Takanami, M. (1973) "DNA sequence restricted by restriction endonuclease AP from *Haemophilus aphirophilus,*" *Nature New Biol.* **246,** 138–140.
19. Old, R., Murray, K. & Roizes, G. (1975) "Recognition sequence of restriction endonuclease III from *Hemophilus influenzae,*" *J. Mol. Biol.* **92,** 331–339.
20. Marvin, D. A. & Hohn, B. (1969) "Filamentous bacterial viruses," *Bacteriol. Rev.* **33,** 172–209.
21. Horiuchi, K., Vovis, G. F. & Zinder, N. D. (1974) "Effect of deoxyribonucleic acid length on the adenosine triphosphatase activity of *Escherichia coli* restriction endonuclease B," *J. Biol. Chem.* **249,** 543–552.
22. Model, P. & Zinder, N. D. (1974) "*In vitro* synthesis of bacteriophage f1 proteins," *J. Mol. Biol.* **83,** 231–251.
23. Hattman, S., Schlagman, S. & Cousens, L. (1973) "Isolation of a mutant of *Escherichia coli* defective in cytosine-specific deoxyribonucleic acid methylase activity and in partial protection of bacteriophage λ against restriction by cells containing the N-3 drug-resistance factor," *J. Bacteriol.* **115,** 1103–1107.
24. Horiuchi, K. & Zinder, N. D. (1972) "Cleavage of bacteriophage f1 DNA by the restriction enzyme of *Escherichia coli* B," *Proc. Nat. Acad. Sci. USA* **69,** 3220–3224.
25. Allet, B. (1973) "Fragments produced by cleavage of λ deoxyribonucleic acid with the *Hemophilus parainfluenzae* restriction enzyme Hpa II," *Biochemistry* **12,** 3972–3977.
26. Boyer, H. W. (1974) "Restriction and modification of DNA: enzymes and substrate," *Fed. Proc.* **33,** 1125–1127.
27. Sadowski, P.D. & Hurwitz, J. (1969) "Enzymatic breakage of deoxyribonucleic acid, II. Purification and properties of endonuclease IV from T4 phage-infected *Escherichia coli,*" *J. Biol. Chem.* **244,** 6192–6198.
28. Ling, V. (1971) "Partial digestion of ^{32}P-fd DNA with T4 endonuclease IV," *FEBS Lett.* **19,** 50–54.
29. Ziff, E. B., Sedat, J. W. & Galibert, F. (1973) "Determination of the nucleotide sequence of a fragment of bacteriophage ϕx174 DNA," *Nature New Biol.* **241,** 34–37.
30. Robertson, H. D., Barrel, B. G., Weith, H. L. & Donelson, J. E. (1973) "Isolation and sequence analysis of a ribosome-protected fragment from bacteriophage ϕx174 DNA," *Nature New Biol.* **241,** 38–40.
31. Linn, S. & Arber, W. (1968) "Host specificity of DNA produced by *Escherichia coli,* X. *In vitro* restriction of phage fd replicative form," *Proc. Nat. Acad. Sci. USA* **59,** 1300–1306.
32. Smith, H. O. & Nathans, D. (1973) "A suggested nomenclature for bacterial host modification and restriction systems and their enzymes," *J. Mol. Biol.* **81,** 419–423.