

# Complementation *in Vitro* by Mutant Restriction Enzymes from *Escherichia coli* K\*

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## SUMMARY

Two mutant strains of *Escherichia coli* K, K-18 lacking restriction activity (endonucleolytic cleavage of foreign DNA), and the other, K-19 lacking both restriction and modification activities (specific DNA methylation that protects against the homologous restriction activity), complement *in vivo* to yield a wild type phenotype. Although neither mutant extract alone binds unmodified DNA, the wild type extract and the mixture of mutant extracts do. Retention of the DNA-enzyme complex on membrane filters was used as an assay to purify the two mutant restriction enzymes. Both of these sediment like the wild type enzyme. Complementation *in vitro* by the two mutant enzymes could be demonstrated by specific DNA binding, cleavage of the unmodified DNA, and restriction-dependent ATP hydrolysis. All of these activities are absent in the individual mutant endonucleases but present in the wild type restriction endonuclease from *E. coli* K. However, both mutant enzymes show an activity that hydrolyzes ATP which is different from that of the wild type enzyme since it does not require unmodified DNA, but is dependent on the presence of S-adenosylmethionine.

Restriction endonucleases are enzymes that cleave double-stranded DNA synthesized in other bacterial strains. They are involved in the phenomenon of host-controlled restriction and modification in which the acceptance or destruction of an entering DNA molecule is dependent on the restriction specificity of the host cell and on certain nonheritable characteristics acquired by the DNA in the cell in which it was replicated. This host-controlled modification appears to be due to specific methylation at certain nucleotide sequences on the DNA which renders it resistant to attack by the corresponding restriction endonuclease from the same cell (1, 2).

The restriction enzymes from the related *Escherichia coli* strains K and B<sup>1</sup> cleave foreign DNA in the presence of Ado-

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<sup>1</sup> The abbreviations used are: K and B, restriction and modification specificities associated with *E. coli* K and *E. coli* B, respectively;  $\lambda$ .0, phage  $\lambda$  grown on a nonmodifying strain;  $\lambda$ .K phage  $\lambda$  grown on a K strain; Ado-Met, S-adenosylmethionine; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; endo-

Met, ATP, and Mg<sup>2+</sup> (3-6). The K restriction enzyme also possesses a specific methylase activity that transfers the methyl groups from Ado-Met to unmodified  $\lambda$  DNA in the absence of ATP and Mg<sup>2+</sup>. The methylated DNA is protected against degradation by the restriction activity of the same enzyme (7). In addition, K- and B-specific restriction endonucleases also have an ATPase activity associated with restriction (2, 8, 9).

Mutants of *E. coli* K and B lacking restriction activity can have two kinds of phenotype: defective only in restriction ( $r^-m^+$ ) or defective in both restriction and modification ( $r^-m^-$ ). One-step and two-step  $r^-m^-$  mutants can be obtained. Genetic experiments with such mutants have led to a three-gene model for restriction and modification in *E. coli*. In its simplest formulation, one gene, *hsdR*, would be primarily responsible for restriction; another one, *hsdS*, for the site specificity of the recognition reaction; and a third one, *hsdM*, for modification (10, 11). The results of Hubacek and Glover suggest that *hsdM* is also required for restriction (12). An oligomeric protein composed of the *hsdR*, *hsdS*, and *hsdM* gene products would therefore have both restriction and modification activities, whereas one composed of the *hsdS* and *hsdM* gene products would only have modification activity. This model is in agreement with the subunit structure of the purified enzymes (1, 6, 13). The restriction endonuclease from *E. coli* K has a molecular weight of approximately 400,000 and is composed of subunits of three different sizes with approximate molecular weights of 135,000, 62,000, and 52,000. This enzyme can recognize specific nucleotide sequences and, depending on the cofactors and incubation conditions, can proceed to cleave or methylate the unmodified DNA (1).

The strains K-18 and K-19 have been shown to complement *in vivo* for restriction.<sup>2</sup> The present study was undertaken in order to determine which subunits are responsible for nucleotide sequence recognition, endonucleolytic activity, DNA methylation, and ATPase activity. In this paper, the purification and properties of the defective restriction enzymes from the mutant strains K-18 with a  $r_K^-m_K^+$  phenotype and a presumed *hsdR\_K^-* genotype, and K-19 with a  $r_K^-m_K^-$  phenotype and presumed *hsdS\_K^-* genotype are described. That K-19 has a *hsdS\_K^-* genotype is indicated by complementation experiments with a  $\lambda$  carrying  $r_B^+m_B^+$ . We have made use of a number of biochemical

nuclease R.K, restriction endonuclease from *E. coli* K; endonuclease R.K-18, restriction endonuclease from K mutant K-18; and endonuclease (endo) R.K-19, restriction enzyme from mutant strain K-19.

<sup>2</sup> W. Arber, unpublished results.

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reactions to demonstrate complementation *in vitro* by these two mutant restriction enzymes.

#### EXPERIMENTAL PROCEDURES

##### Materials

**Bacteria and Bacteriophages**—The wild type *Escherichia coli* K used was the 1100 KLF1 endonuclease I-deficient strain from the collection of Dr. M. Meselson, Harvard University. It is a partial diploid carrying two copies of the K restriction and modification genes. The *E. coli* strains K-18 and K-19 are the 802 and 803 of Wood (14) and have the phenotype  $r_K^-m_K^+$  and  $r_K^-m_K^-$ , respectively. The  $\lambda$  stocks used were the wild type of Kaiser (15),  $\lambda$ CI, a clear plaque-forming mutant, and  $\lambda$ CI857*sus*S7.

**DNA Preparations**—Stocks of modified [<sup>3</sup>H]thymine  $\lambda$ .K phage were prepared in radioactive medium by ultraviolet induction of the lysogen C600 *thy*<sup>-</sup>*TRT5*<sup>R</sup>( $\lambda$ ) or by thermal induction of W1485(P1) ( $\lambda$ CI857*sus*S7) which carries both K and P1 restriction and modification markers. Unmodified  $\lambda$ .0 [<sup>32</sup>P]phage was prepared in radioactive medium by thermal induction of 803 ( $\lambda$ CI857*sus*S7) which is  $r_K^-m_K^-$ . Phages were purified and DNA extracted as described earlier (3). Calf thymus DNA was purchased from Sigma Chemical Co.

**Other Materials**—Ado-Met, ATP, and other nucleotides were obtained from Sigma. The commercial Ado-Met was further purified by elution from Bio-Rex 70 with 4 N acetic acid. TES was purchased from Calbiochem. Sephadex G-50 was obtained from Pharmacia Chemicals, DEAE-cellulose DE52 and phosphocellulose P11 from Whatman Biochemicals, and hydroxylapatite from Clarkson Chemical Co. The nitrocellulose filters used in the binding assay were Sartorius SM 11306 with a diameter of 25 mm. The polyethyleneiminecellulose thin layer plates were Macherey-Nagel Polygram CEL300PEI. [<sup>3</sup>H]ATP and [<sup>3</sup>H]Ado-Met were from the Radiochemical Center, Amersham, United Kingdom.

##### Methods

**Binding Assays for Restriction Activity**—A routine assay for the wild type enzyme making use of the binding of the DNA-enzyme complex to nitrocellulose filters has been developed. In assays of less purified fractions, an excess of calf thymus DNA was added to suppress nonspecific DNA binding. The detailed procedure was published earlier (16). To assay for a mutant restriction enzyme, the sample (e.g. of endonuclease R.K-18) was incubated in the standard binding reaction mixture in the presence of 10  $\mu$ l (approximately 25  $\mu$ g of protein) of the DEAE-fraction of the complementing activity (e.g. endonuclease R.K-19). To assay the fractions from the glycerol gradients, the purified glycerol gradient fraction of the complementing enzyme had to be used to eliminate nonspecific DNA binding. No calf thymus DNA was present in the assays of this final step in the purification. Except for the addition of the complementing enzyme, the conditions of the binding assay were identical with those used with the wild type activity.

An enzyme unit is defined as the amount of protein that will specifically bind 5% of the  $\lambda$ .0 DNA in a reaction mixture containing  $6.4 \times 10^8$  phage equivalents of  $\lambda$ .0 DNA and 125  $\mu$ g of calf thymus DNA during a 10-min incubation at 30°.

**Neutral Sucrose Sedimentation**—The specific endonucleolytic activity was measured by sedimentation analysis in neutral sucrose gradients (3).

**ATPase Assay**—The ATPase activity of the wild type and mutant restriction enzymes was estimated as described previously (8).

**DNA Methylase Assay**—The DNA modification methylase activity associated with these restriction enzymes was measured according to the method of Haberman *et al.* (7).

**Purification Procedure for Mutant Restriction Endonucleases**—All cells were grown to a density of 3 to 6  $\times 10^8$  cells per ml in tryptone broth supplemented with yeast extract. The cells were harvested and stored at -80° until used.

**Endonuclease R.K-18**—Frozen K-18 cells, 102 gm, were suspended in 570 ml of cold 0.01 M Tris (pH 7.4), 10<sup>-4</sup> M EDTA, 2  $\times 10^{-3}$  M mercaptoethanol, and 2  $\times 10^{-4}$  M MgCl<sub>2</sub> (Buffer A). All operations were performed at 4°. The cells were disrupted by using the Dyno Laboratory mill, type KDL (Willy Bachofen Maschinenfabrik, Basel, Switzerland). Cell suspension, 160 ml, and 255 ml of dry glass beads (0.1 mm diameter) were loaded into the 300-ml

batch container each time and run at 2,000 rpm for 3 min. Ninety to ninety-five per cent of the cells were disrupted. The extract was centrifuged at low speed and then at 30,000 rpm for 2 hours in the International A-170 rotor.

The high speed supernatant was subjected to fractional precipitation by the addition of solid ammonium sulfate taking cuts at 0 to 35% and 35 to 55% saturation. The material that precipitated between 35 and 55% saturation was dissolved in 100 ml of 0.02 M potassium phosphate (pH 7.0), 10<sup>-4</sup> M EDTA and 2  $\times 10^{-3}$  M mercaptoethanol (Buffer B) and dialyzed overnight against Buffer B. It was then applied to a column (2.5  $\times$  45 cm) of DEAE-cellulose DE52 (bed volume 150 ml) which had been equilibrated against Buffer B. The column was washed with 300 ml of Buffer B and eluted with a 1000-ml linear gradient of Buffer B running from 0.05 M to 0.3 M potassium phosphate. Fractions of 25 ml were collected and aliquots were assayed. The activity was eluted at a potassium phosphate concentration between 0.14 M and 0.16 M.

The pooled DEAE-fractions were dialyzed against Buffer B and chromatographed on a column (2.5  $\times$  10 cm) (bed volume 40 ml) of Whatman phosphocellulose P11 equilibrated against the same buffer. The column was washed with 50 ml of Buffer B and eluted with a 250-ml linear gradient of Buffer B running from 0.05 M to 0.3 M potassium phosphate. Fractions of 10 ml were collected, and the activity emerged in the range 0.16 M to 0.22 M potassium phosphate.

The pooled phosphocellulose fractions were dialyzed and concentrated to 5 ml in an Amicon ultrafiltration cell No. 52 with a PM-10 filter. It was layered in aliquots of 2.5 ml on 30 ml, 10 to 25% glycerol gradients made up in 0.01 M potassium phosphate (pH 7.5), 10<sup>-4</sup> M EDTA, and 5  $\times 10^{-5}$  M dithiothreitol and centrifuged in an International SB-110 rotor at 25,000 rpm for 20 hours at 4°. Thirty fractions of 1 ml were collected and the activity was found as a broad band sedimenting in Fractions 12 to 20.

**Endonuclease R.K-19**—Frozen K-19 cells, 120 g, were disrupted in the Dyno mill, centrifuged at low and high speed, and fractionated with ammonium sulfate in the same manner as described for K-18 above.

The 35 to 55% ammonium sulfate fraction was chromatographed on a column (2.5  $\times$  19 cm) of DEAE-cellulose (bed volume 150 ml) which had been equilibrated against Buffer B. The column was washed with 200 ml of Buffer B and eluted with a 600-ml linear gradient of Buffer B running from 0.05 M to 0.3 M potassium phosphate. Fractions of 25 ml were collected and the activity came off at a potassium phosphate concentration between 0.14 M and 0.17 M.

The DEAE-fraction was dialyzed against Buffer B, and applied to a column (2.5  $\times$  8.5 cm) of hydroxylapatite (bed volume 50 ml) which had been equilibrated against the same buffer. The column was washed with 50 ml of Buffer B and eluted with a 250-ml linear gradient going from 0.05 M to 0.3 M potassium phosphate. Fractions of 5 ml were collected, and the enzyme came off at a potassium phosphate concentration between 0.07 M and 0.11 M.

The hydroxylapatite fraction was dialyzed and concentrated to 9 ml using the Amicon ultrafiltration cell with a PM-10 filter. The concentrate was loaded in 3-ml portions on 30 ml of 10 to 25% glycerol gradients and centrifuged at 25,000 rpm for 20 hours at 4°. Thirty fractions of 1 ml were collected. The activity appeared in Fractions 17 to 20.

For some experiments R.K-19 was further purified on 4% analytical polyacrylamide gels (0.5  $\times$  6 cm) run in Tris-barbiturate buffer at pH 7.6. Electrophoresis was performed at 4° on the glycerol gradient fraction containing 70  $\mu$ g of protein and the gel was sliced into 1-mm slices. Fractions of three slices each were suspended in 0.25 ml of 0.01 M potassium phosphate buffer (pH 7.5), 10<sup>-4</sup> M EDTA, 5  $\times 10^{-5}$  M dithiothreitol, and 10% glycerol and shaken overnight at 4°. The position of the enzyme was localized by assaying an aliquot in the usual binding assay. The slices containing the enzyme were macerated and centrifuged to remove the gel particles and the supernatant used as the purified enzyme.

#### RESULTS

**In Vitro Complementation with Crude Extracts**—Complementation *in vitro* was studied with extracts of *E. coli* K-18 ( $r_K^-m_K^+$ ) and *E. coli* K-19 ( $r_K^-m_K^-$ ). The 35 to 55% ammonium sulfate fractions of these mutants were prepared and complementation shown with the binding assay. In this assay,

TABLE I  
DNA binding by crude extracts

Reaction mixtures with a volume of 0.25 ml contained 25  $\mu$ moles of TES (pH 8.0), 0.7  $\mu$ mole of EDTA, 1.6  $\mu$ moles of  $MgCl_2$ , 3  $\mu$ moles of 2-mercaptoethanol, 0.1  $\mu$ mole of ATP, 0.02  $\mu$ mole of Ado-Met,  $3.5 \times 10^9$  phage equivalents (4,000 cpm) of  $\lambda$ .K [ $^3H$ ]DNA,  $3 \times 10^9$  phage equivalents (14,000 cpm) of  $\lambda$ .0 [ $^{32}P$ ]DNA, and 100  $\mu$ g of calf thymus DNA. Additions of enzyme extracts were as follows: 55  $\mu$ g of K, 52.5  $\mu$ g of K-18, and 43  $\mu$ g of K-19. These were all 35% to 55% ammonium sulfate fractions. After incubation for 2 min at 30°, the reactions were terminated by the addition of 0.03 ml of 0.5 M EDTA, pH 8. The mixtures were then filtered through nitrocellulose filters.

<i>E. coli</i> extract	ATP and Ado-Met omitted		Complete reaction mixture	
	$\lambda$ .K [ $^3H$ ]DNA <sup>a</sup>	$\lambda$ .0 [ $^{32}P$ ]DNA <sup>a</sup>	$\lambda$ .K [ $^3H$ ]DNA <sup>a</sup>	$\lambda$ .0 [ $^{32}P$ ]DNA <sup>a</sup>
K ( $r_K^+m_K^+$ )	5.40	2.20	4.60	9.20
K-18 ( $r_K^-m_K^+$ )	4.40	2.40	0.74	0.64
K-19 ( $r_K^-m_K^-$ )	1.70	1.67	1.42	1.70
K-18 + K-19	1.20	1.40	1.20	6.80

<sup>a</sup> Expressed as per cent of input DNA bound to filters.

the enzyme is added to a reaction mixture containing differentially labeled modified and unmodified  $\lambda$  DNAs, Ado-Met, ATP, and  $Mg^{2+}$ . After a brief incubation it is passed through a nitrocellulose filter, and restriction activity is detected as specific binding of unmodified  $\lambda$ .0 DNA to the filter (17). These complementation results were similar to those reported previously by Linn and Arber (4) from infectivity experiments with extracts from  $r_B^-m_B^+$  and  $r_B^-m_B^-$  strains. In the experiment summarized in Table I, 100  $\mu$ g of calf thymus DNA were added to each reaction mixture to suppress the nonspecific binding of other proteins to the  $\lambda$  DNAs. The extract from *E. coli* K was able to bind twice as much unmodified  $\lambda$ .0 DNA as  $\lambda$ .K DNA. Although neither of the two mutant extracts specifically bound unmodified  $\lambda$  DNA, when combined they bound 5 times more  $\lambda$ .0 DNA than  $\lambda$ .K DNA. In both the wild type extract and the complementing mixture of K-18 and K-19 extracts, the specific binding was dependent on the presence of Ado-Met and ATP. However, these results should only be considered qualitative since the presence of calf thymus DNA also suppresses to a certain extent the specific binding of the restriction enzyme to the unmodified  $\lambda$  DNA. From our unpublished results there is evidence that calf thymus DNA may possess restriction sites for the *E. coli* K restriction endonuclease. These results indicated that mixing together the two mutant extracts produced an activity similar in properties and requirements to that of the restriction endonuclease from *E. coli* K.

**Purification of Mutant Restriction Enzymes**—By using partially purified preparations of one mutant enzyme and the filter binding assay, the complementing restriction enzyme could be purified. The purified mutant enzyme could in its turn be used to purify the other one. Unless otherwise indicated, the activity of a mutant restriction endonuclease is operationally defined as its ability to specifically bind unmodified  $\lambda$  DNA in the presence of an excess of the complementing enzyme. Table II gives a summary of the two purifications, the details appear under "Methods."

The endonuclease R.K-18 purifies in a manner identical with that of the wild type restriction enzyme and has been purified

TABLE II  
Purification of mutant restriction enzymes

	Endonuclease R.K-18 ( $r_K^-m_K^+$ )			Endonuclease R.K-19 ( $r_K^-m_K^-$ )		
	Volume	Total protein	Specific activity	Volume	Total protein	Specific activity
High speed supernatant	500 ml	5500 mg	0.36 units/mg	650 ml	5330 mg	0.16 units/mg
Ammonium sulfate precipitation	100 ml	3650 mg	2.7 units/mg	150 ml	3000 mg	2.52 units/mg
DEAE-fraction	125 ml	350 mg	5.25 units/mg	130 ml	325 mg	4.8 units/mg
Phosphocellulose fraction (concentrated)	75 ml	8.25 mg	33.3 units/mg	8 ml	47.2 mg	8.9 units/mg
Glycerol gradient fraction	10 ml	0.17 mg	916 units/mg	15 ml	5.25 mg	63.4 units/mg

TABLE III

Complementation by mutant restriction enzymes in DNA binding

The complete reaction mixture contained in a 0.25 ml volume: 25  $\mu$ moles of TES (pH 8.0), 0.07  $\mu$ mole of EDTA, 1.6  $\mu$ moles of  $MgCl_2$ , 3  $\mu$ moles of mercaptoethanol, 0.1  $\mu$ mole of ATP, 0.02  $\mu$ mole Ado-Met, and  $\lambda$ .K [ $^3H$ ]DNA ( $2.22 \times 10^9$  (20,100 cpm) phage equivalents) and  $\lambda$ .0 [ $^{32}P$ ]DNA ( $1.97 \times 10^9$  (1,870 cpm) phage equivalents) as indicated on the table. Endonuclease R.K (0.06  $\mu$ g) or 0.25  $\mu$ g of endonuclease R.K-18 or 3.5  $\mu$ g of R.K-19 were added. Components were omitted from the assay mixture as indicated in the table. Incubation was done at 30° for 2 min and 0.03 ml of 0.5 M EDTA was then added. The samples were then passed through nitrocellulose filters as described under "Methods."

Enzyme added	Components omitted from complete reaction	Phage equivalents of DNA retained $\times 10^3$	
		$\lambda$ .K [ $^3H$ ]DNA	$\lambda$ .0 [ $^{32}P$ ]DNA
Endo R.K.	None	3.0	19.4
Endo R.K-18	None	2.2	0.8
Endo R.K-19	None	1.1	0.4
Endo R.K-18 + Endo R.K-19	None	2.3	19.3
Endo R.K-18 + Endo R.K-19	Ado-Met, ATP, $Mg^{2+}$	1.0	0.3
Endo R.K-18 + Endo R.K-19	Ado-Met, ATP	3.0	1.0
Endo R.K-18 + Endo R.K-19	Ado-Met	2.0	0.2
Endo R.K-18 + Endo R.K-19	ATP	7.3	4.0
Endo R.K-18 + Endo R.K-19	Mg	2.0	0.5

close to 2500-fold. On the other hand, the endonuclease R.K-19 does not bind to phosphocellulose, and only weakly to hydroxylapatite, and in these respects is very different to the wild type and K-18 proteins. This has resulted in its being purified only 400-fold and analysis on polyacrylamide gels shows the presence of a number of contaminating proteins. Both mutant restriction endonucleases sediment similarly to the wild type enzyme in glycerol gradients and thus are not enzyme fragments. The glycerol gradient fractions were stored at -40° and were stable for 6 months. In all of the following experiments, the glycerol gradient fraction was used routinely unless otherwise indicated.

**DNA Binding**—The ability of the purified enzymes to bind unmodified DNA was measured in the experiment shown in Table III. The wild type enzyme specifically bound almost

Endonuclease R.K-19 (μg)	Specific activity (units/mg)
0.16	0.16
0.32	2.52
0.64	4.8
1.28	8.9
2.56	63.4

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total volume:  
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(m) phage  
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K-19 were  
mixture as  
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λ.0 [ <sup>32</sup> P]-DNA	Equivalents retained × 10 <sup>3</sup>
19.4	0.8
0.8	0.4
0.4	19.3
19.3	0.3
0.3	1.0
1.0	0.2
0.2	4.0
4.0	0.5

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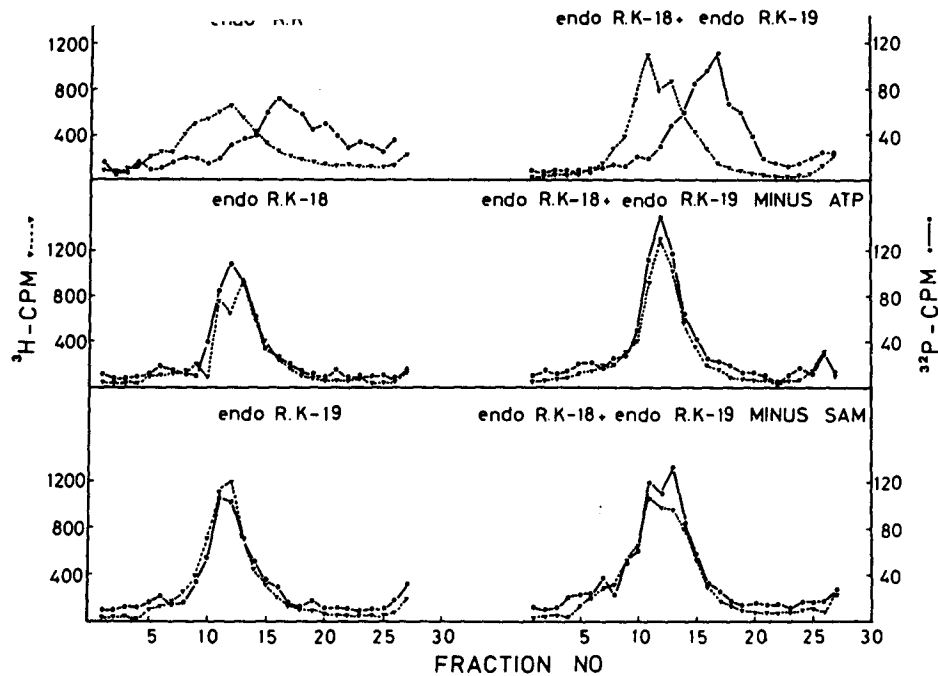


FIG. 1. Complementation by mutant restriction enzymes in endonuclease activity. The reaction mixtures were the same as those described in Table I except that the input of  $\lambda$ .K [<sup>3</sup>H]DNA and  $\lambda$ .0 [<sup>32</sup>P]DNA was  $2.4 \times 10^9$  (22,000 cpm) and  $1.79 \times 10^9$  (1,700 cpm) phage equivalents, respectively. Enzyme additions were:

0.12 μg of endonuclease R.K or 0.36 μg of endonuclease R.K-18 or 7 μg of endonuclease R.K-19. The incubation was carried out at 30° for 20 min and stopped by the addition of 0.03 ml 0.5 M EDTA, pH 8.0. The samples were then subjected to sedimentation analysis in neutral sucrose gradients.

TABLE IV  
DNA modification methylase activity

The reaction mixtures contained in a volume of 0.15 ml: 15 μmoles of TES (pH 8.0), 0.03 μmole of EDTA, 1.5 μmoles of mercaptoethanol, 0.38 nmole of [methyl-<sup>3</sup>H]Ado-Met (13 Ci per mmole),  $4 \times 10^{10}$  phage units of modified or  $3.4 \times 10^{10}$  phage units of unmodified  $\lambda$  DNA and approximately 0.3 μg of endonuclease R.K, 0.36 μg of endonuclease R.K-18 or 7 μg of the K-19 enzyme. After incubation at 37° for 18 hours  $\lambda$  [<sup>32</sup>P]DNA (1950 cpm) and 250 μg of calf thymus DNA were added, the DNA was precipitated by addition of 1 ml ice-cold 10% trichloroacetic acid-0.01 M sodium pyrophosphate-1 M NaCl. The samples were left in ice for 30 min before centrifuging at 6000 × g for 15 min. The supernatants were discarded and the pellets were dissolved in 0.4 ml 0.5 M NH<sub>4</sub>OH with vigorous agitation. The solution was extensively dialyzed against 0.01 M Tris (pH 8.0)-0.01 M NaCl- $2 \times 10^{-4}$  M EDTA and reprecipitated as described above. Each precipitate was collected on a glass fiber filter and washed with 10 ml of cold trichloroacetic acid, sodium pyrophosphate, and NaCl followed by 10 ml methanol. The filters were then counted in Aquasol.

Enzymes	DNA substrate	Methyl groups per DNA molecule
Endo R.K	$\lambda$ .K	<0.1
Endo R.K-18	$\lambda$ .K	<0.1
Endo R.K-19	$\lambda$ .K	0.6
Endo R.K-18 + Endo R.K-19	$\lambda$ .K	0.3
Endo R.K	$\lambda$ .0	6.6
Endo R.K-18	$\lambda$ .0	5.1
Endo R.K-19	$\lambda$ .0	0.7
Endo R.K-18 + Endo R.K-19	$\lambda$ .0	4.8

all of the unmodified  $\lambda$ .0 DNA in a complete system, but no such effect was observed with the endonucleases R.K-18 or R.K-19 by themselves. However, when the two mutant endonucleases were mixed together along with the DNAs and the

cofactors and passed through a membrane filter, specific retention of almost all of the  $\lambda$ .0 DNA was observed. The cofactor requirements for specific DNA binding by the complementing mixture of endonucleases R.K-18 and R.K-19 was identical with that of the wild type restriction enzyme (*i.e.* Ado-Met, ATP, and Mg<sup>2+</sup>). As is the case with the wild type enzyme, a certain amount of nonspecific binding was observed if ATP was omitted.

**Restriction Endonuclease Activity**—The wild type restriction endonuclease readily cleaves unmodified  $\lambda$ .0 DNA by making a limited number of double-stranded scissions in the presence of Ado-Met, ATP, and Mg<sup>2+</sup>. It has no effect on  $\lambda$ .K DNA (3).

In the experiment shown on Fig. 1, the effect of the three endonucleases on the sedimentation rate of a mixture of  $\lambda$ .K [<sup>3</sup>H]-DNA and  $\lambda$ .0 [<sup>32</sup>P]DNA was determined on neutral sucrose gradients. As was expected, the wild type endonuclease specifically degraded unmodified  $\lambda$ .0 DNA while neither of the two mutant endonucleases had any effect on it. When the endonucleases R.K-18 and R.K-19 were present together with all of the cofactors, the unmodified  $\lambda$ .0 DNA was specifically cleaved. In the absence of either Ado-Met or ATP, no reaction was observed. Both in its specific double-stranded cleavage of unmodified DNA as well as in its requirements for Ado-Met and ATP in this reaction, the combined mutant endonucleases resembled the wild type enzyme.

**DNA Modification Methylase Activity**—It has been demonstrated that the restriction endonuclease from *E. coli* K has a DNA modification methylase activity which transfers an average of 8 methyl groups from Ado-Met to unmodified  $\lambda$ .0 DNA. Incubations carried out with larger amounts of enzyme can lead to a maximum incorporation of 10 to 15 methyl groups per  $\lambda$  molecule. This reaction requires only Ado-Met, and the *in vitro* modified DNA is rendered resistant to cleavage by the same enzyme in the complete restriction system (7). The number of methyl groups incorporated *in vitro* agrees well with the figure



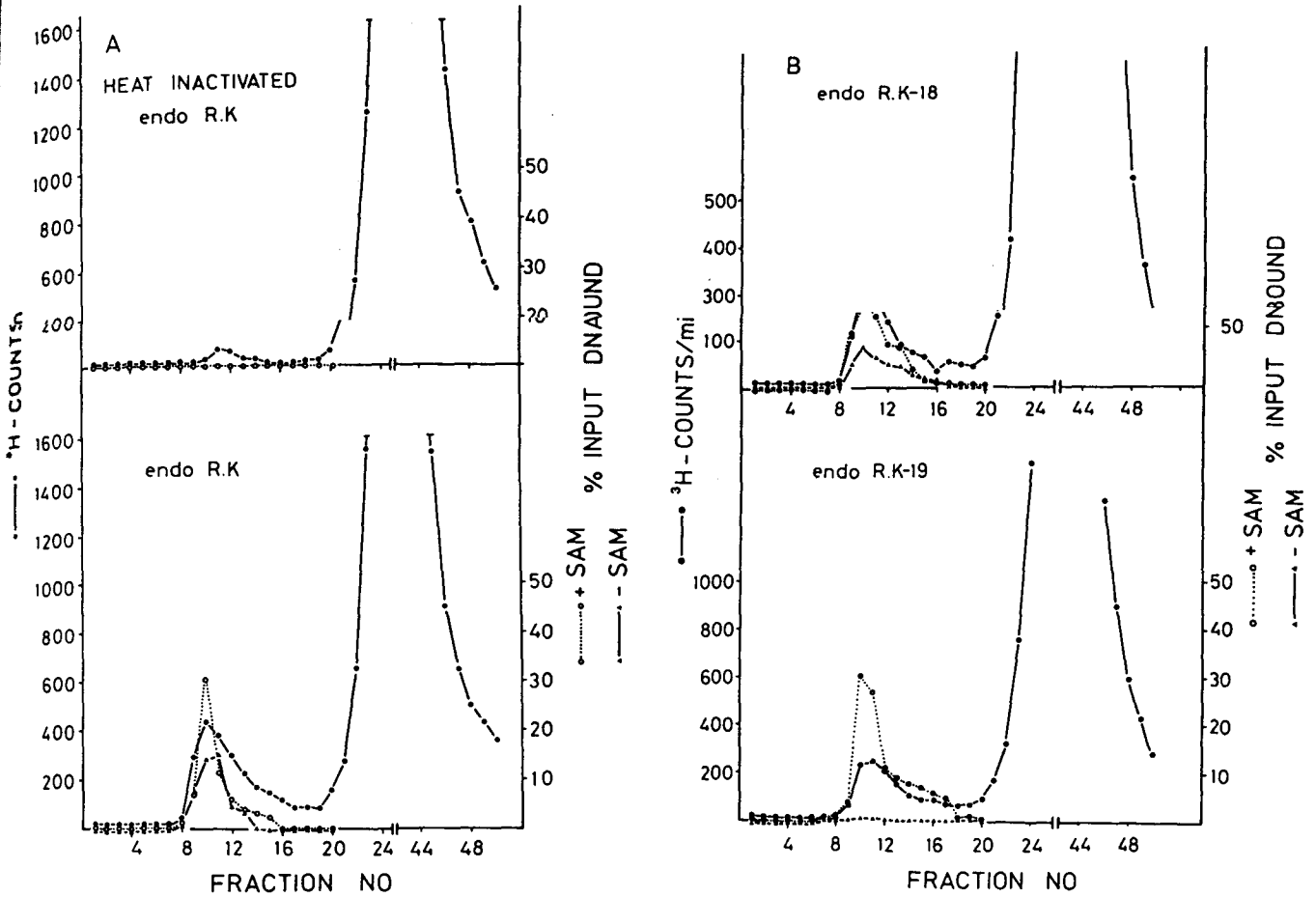


Fig. 2. Gel filtration of Ado-Met-enzyme complexes. The reaction mixtures contained in a volume of 0.1 ml: 10  $\mu$ moles of TES (pH 8.0), 0.03  $\mu$ mole of EDTA, 0.64  $\mu$ mole of  $MgCl_2$ , 1.2  $\mu$ moles of mercaptoethanol, and 0.435 nmole of [methyl-<sup>3</sup>H]Ado-Met (11.4 Ci per mmole). The amount of enzyme added to each sample was 37  $\mu$ g of endonuclease R.K., 15  $\mu$ g of endonuclease R.K-18, and 45  $\mu$ g of endonuclease R.K-19. The samples were incubated at 30° for 2 min and 0.01 ml 0.5 M EDTA (pH 8.0) was then added. Each

sample was then applied to a Sephadex G-50 column (0.5  $\times$  24 cm) which had been equilibrated with 0.01 M Tris (pH 7.4), 10<sup>-4</sup> M EDTA, and 2  $\times$  10<sup>-3</sup> M mercaptoethanol (Buffer C) containing 10% glycerol. The column was run with Buffer C at a flow rate of 12 ml per hour at 4° and fractions of 0.25 ml were collected. Aliquots of 0.05 ml from the first 20 fractions were assayed for DNA binding in the presence and absence of Ado-Met. Samples of 0.05 ml were also counted for tritium in 3 ml of Aquasol.

ever, if Ado-Met was omitted in the enzyme assays, 50% of the activity could still be detected indicating that the Ado-Met bound to the enzyme appears to be sufficient to allow specific binding to unmodified DNA without further addition of Ado-Met. The enzyme complex carrying the tritium label was concentrated, sodium dodecyl sulfate was added to a final concentration of 0.4%, and the sample was analyzed by thin layer chromatography. All of the tritium was present as Ado-Met indicating that the methyl group of the Ado-Met was neither covalently bound to the enzyme nor had undergone any chemical change.

Similar experiments were done with the mutant enzymes. The results are shown in Fig. 2B. As mentioned previously, the activity of the mutant restriction enzymes is measured in terms of its ability to bind specifically unmodified DNA in the presence of the complementing enzyme. With the endonuclease R.K-18, a tritium peak emerged in the void volume along with the enzyme activity (measured with the complete assay system). The percentage of DNA binding was higher in the case of endonuclease R.K-18 because a lower concentration of unmodified DNA was present in the assays. If Ado-Met was omitted from

the assay reaction, approximately 50% of the activity could still be detected. This indicated that the mutant enzyme from strain K-18 was able to bind Ado-Met and use it in conjunction with the complementing endonuclease R.K-19 to bind unmodified DNA.

In the case of endonuclease R.K-19, a tritium peak appeared in the void volume along with the enzyme activity. If the Ado-Met was omitted in the assays, no enzyme activity was detected. It must be stressed however, that the Ado-Met binding observed may very well be due to one of the contaminating proteins and not to the mutant enzyme itself. What is clear is that even if the mutant enzyme from K-19 does bind Ado-Met, it is unable to use it in combination with the endonuclease R.K-18.

#### DISCUSSION

The wild type and mutant restriction enzymes of *E. coli* K have been compared by studying a number of different reactions: the specific binding and endonucleolytic cleavage of unmodified  $\lambda$  DNA, DNA methylation, and the hydrolysis of ATP during the restriction reaction. The restriction endonuclease R.K-18 (from the r<sub>K</sub><sup>-</sup>m<sub>K</sub><sup>+</sup> strain) purifies in a manner identical with that of

the wild type enzyme. On the other hand, the enzyme from the  $r_K^-m_K^-$  strain K-19 which presumably lacks the site recognition function is quite different insofar as it is unable to bind to phosphocellulose and is weakly bound to hydroxylapatite. All three enzymes have the same size of approximately 12 S.

Neither of the two mutant endonucleases bound to unmodified  $\lambda$ 0 DNA. This is unexpected since the endonuclease R.K-18 should have an intact recognition subunit. These results would indicate that both the restriction and recognition subunits are necessary for formation of the specific complex that is bound to the nitrocellulose filters. At the same time, it suggests that the specific binding seen with the wild type endonuclease is associated with the cleavage reaction rather than with just recognition of the host specificity sites on the DNA.

The endonuclease and methylase activities of the two mutant enzymes are in accordance with the *in vivo* observations; neither of the two can cleave unmodified DNA, and the endonuclease R.K-18 but not the endonuclease R.K-19 can methylate unmodified DNA.

The hydrolysis of ATP is an unexplained property of the restriction endonuclease but it is known that it requires the endonucleolytic reaction to take place. Two hypotheses have been advanced to explain this ATPase: (a) ATP hydrolysis may provide the activation energy for some conformational change required for the specific recognition of unmodified DNA; or (b) the cleavage of unmodified DNA yields an altered enzyme that catalyzes the ATP hydrolysis. It is interesting to note that the ATPase characteristic of the wild type enzyme is absent from both mutant enzymes, neither of which can restrict. These enzymes however do have an ATPase activity which is dependent solely on the presence of Ado-Met.

Complementation *in vitro* can be readily demonstrated with the mutant enzymes from strains K-18 and K-19. When both proteins are present together, there is specific binding to unmodified DNA, double-stranded scission of the DNA, and ATP breakdown—all of these showing the same requirements as the wild type enzyme. None of these results allow us to distinguish between complementation *in vitro* due to subunit exchange between the two mutant proteins and a reaction consisting of at least two steps each one catalyzed by one of the mutant enzymes. Several experiments have been done in an effort to isolate wild type enzyme by chromatography on phosphocellulose columns of a mixture of endonucleases R.K-18 and R.K-19 that had been incubated with or without cofactors under a variety of conditions. No wild type activity was observed. Attempts to isolate a DNA intermediate after incubation with one of the mutant enzymes and then incubating it with the complementing enzyme have yielded ambiguous results. Further experiments are in progress with these enzymes and those from other mutants to elucidate this matter.

Finally, our experiments show that the wild type enzyme is able to bind Ado-Met. The Ado-Met-enzyme complex formed is active in the DNA binding reaction without the addition of further Ado-Met. The same is true for endonuclease R.K-18 which also forms a complex with Ado-Met, and in combination with endonuclease R.K-19 can bind to unmodified DNA without added Ado-Met. The endonuclease R.K-19 may or may not bind Ado-Met, but it is unable to use it for DNA binding. In any case, the Ado-Met bound to endonuclease R.K-18 is sufficient to allow complementation with the K-19 enzyme to take place. Whether the Ado-Met bound to the restriction enzyme is then transferred to the unmodified DNA as one of the steps of the restriction reaction or is only required as an allosteric effector is one of the matters under study at the present time.

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