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# Methylase Activities from *Haemophilus influenzae* that Protect *Haemophilus parainfluenzae* Transforming Deoxyribonucleic Acid from Inactivation by *Haemophilus influenzae* Endonuclease R

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Specific methylases that have the properties of deoxyribonucleic acid (DNA) modification enzymes have been isolated from *Haemophilus influenzae* strain Rd. Two activities (methylase IIa and methylase III) were found to protect transforming DNA of *H. parainfluenzae* from the action of *H. influenzae* restriction enzymes. To determine the specificity of the protection, a procedure based on biological activity was developed for the separation and purification of the restriction endonucleases from *H. influenzae* strain Rd. Two endonuclease R activities presumably corresponding to *Hind II* and *Hind III* (P. H. Roy and H. O. Smith, 1973; H. O. Smith and K. W. Wilcox, 1970) were characterized by differences in their chromatographic properties, ability to attack T7 DNA, and inactivation of the transforming activity of different markers of *H. parainfluenzae* DNA. One endonuclease R enzyme (*Hind II*) attacked T7 DNA and was found to inactivate the dalacin resistance marker (<0.01% activity remaining) with only a slight effect on the streptomycin resistance marker (83% activity remaining). Methylase IIa treatment protected 40% of the dalacin resistance marker of *H. parainfluenzae* DNA from inactivation by *Hind II*. The other restriction activity (*Hind III*) was inert towards T7 DNA and inactivated the streptomycin resistance marker of *H. parainfluenzae* DNA (<0.01% activity remaining) without any effect on the dalacin resistance marker. The methylation of *H. parainfluenzae* DNA accomplished by methylase III protected 60% of the transforming activity of the streptomycin resistance marker of *H. parainfluenzae* DNA from the action of *Hind III*.

The deoxyribonucleic acid (DNA) modification and restriction enzymes not only provide a model system for specific protein-DNA interactions but are also useful tools in molecular biology. With the discovery by Arber and Dussoix (1, 3) that restriction and modification of DNA are a function of the methylation of DNA, it became clear that methylation of specific bases can protect against restriction enzymes that may be carried in the same cell. Our major interest is in the characterization of the restriction-modification enzymes in a system in which the biological properties of these enzymes can be followed.

In this communication we describe the separation and purification of two restriction enzymes from *Haemophilus influenzae* and the isolation and purification of the specific methylases that protect DNA from the action of these restriction enzymes.

## MATERIALS AND METHODS

*H. influenzae* strain Rd com$^{-10}$ was used for the isolation and purification of the restriction endonucleases *H. influenzae* dII (*Hind II*) and *H. influenzae* dIII (*Hind III*) and the DNA modification methylases. Rd com$^{-10}$ was obtained as a competence-deficient mutant of strain Rd but was found to lack the major exonuclease of strain Rd (9). *H. parainfluenzae* (strain colony 14) was originally obtained from G. Leidy and was used as a recipient in transformation. *H. parainfluenzae* StrNovDalEryNal used as a source of transforming *H. parainfluenzae* DNA is a derivative of the wild-type strain that received five antibiotic resistance markers by transformation: streptomycin (*str*), novobiocin (*nov*), dalacin (*dal*), erythromycin (*ery*), and nalidixic acid (*nal*). Phage T7 grown in *Escherichia coli* B strain was used as a source of phage T7 DNA. *H. influenzae* bacteriophage S2 was obtained from a *Haemophilus* strain isolated from a patient at the University of Pennsylvania Hospital. *H. influenzae* 25S used as a host of

phage S2 is an $r^-$ $m^-$ segregant of the wild-type strain Rd.

**Media.** Brain heart infusion (Difco) supplemented with 2 μg of nicotinamide adenine dinucleotide per ml (Nutritional Biochemicals Corp.) and 10 μg of hemin per ml (Eastman Kodak) was used for the growth of *H. influenzae*. *H. parainfluenzae* was grown in brain heart infusion broth medium obtained from Baltimore Biological Laboratories and was supplemented with 2 μg of nicotinamide adenine dinucleotide per ml.

**Antibiotics.** Streptomycin sulfate was obtained from Nutritional Biochemicals Corp. and was used at a final concentration of 200 μg/ml; erythromycin (Eli Lilly and Co.) was dissolved in 50% (vol/vol) ethanol and was used at a final concentration of 10 μg/ml; novobiocin (Upjohn) dissolved in water was used at a final concentration of 10 μg/ml; nalidixic acid (Sterling Winthrop) was dissolved in 0.01 N NaOH and used at a final concentration of 5 μg/ml; dalacin (Upjohn) dissolved in 50% (vol/vol) ethanol was used at a final concentration of 8 μg/ml.

**Preparation of DNAs.** Bacterial DNA species for transformation and methylation studies were obtained using the procedure of Goodgal and Herriot (8).

Bacteriophage S2 DNA was prepared by the following procedure. Lysogenic bacteria 25S Rd (S2) were grown at 37 C to a concentration of $5 \times 10^8$ cells/ml. The cells were induced with mitomycin C (added to give a final concentration of 0.1 μg/ml) by incubation at 37 C with vigorous aeration for 120 to 150 min. To the lysate were added pancreatic deoxyribonuclease and ribonuclease to a final concentration of 10 μg/ml and 25 μg/ml, respectively. Unlysed cells and debris were removed by centrifugation at 5,000 rpm for 15 min in a GSA rotor in a Sorvall RC-2 centrifuge. The supernatant fluid was made 0.5 M in NaCl and polyethylene glycol 6000 (Matheson, Coleman and Bell) was added to give a final concentration of 5%. The phage was precipitated after incubation overnight at 4 C. The phage pellet was collected by centrifugation at 8,000 rpm in a GSA rotor for 10 min and resuspended in 20 ml of M IV medium (10). Phage DNA was extracted with an equal volume of cold redistilled phenol (Baker reagent) freshly dissolved in SSC (0.015 M NaCl, 0.01 M sodium citrate). The DNA was diluted to a volume of 20 ml in SSC and then dialyzed overnight with two changes of 1,000 ml of SSC to remove phenol.

Phage T7 DNA was purified from TCG medium lysates of *E. coli* B (12) by differential centrifugation. The TCG medium contained: 1.0 ml of a 5% solution of vitamin-free Casamino Acids (Difco); 1.0 ml of 10% glucose solution; 0.20 ml of 25% NaCl solution; 0.16 ml of 0.1 M $Na_2SO_4$; 0.32 ml of 0.1 M $NaH_2PO_4$; 0.10 ml of 1.0 M $MgSO_4$; 0.14 ml of 1.0 M $CaCl_2$; 0.03 ml of 0.01 M $FeCl_3$; 100.0 ml of 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.3.

The DNA was extracted with an equal volume of cold redistilled phenol dissolved in NaCl-Tris buffer (0.05 M NaCl, 0.01 M Tris-hydrochloride, pH 7.4) and then dialyzed for 20 h against three changes of 500 ml of the above buffer. In general, three 20-min extractions were used.

Phage T7 DNA labeled with [$^3$H]thymine was prepared from phage grown in TCG medium containing [$^3$H]thymidine, 1 μCi/ml, by the procedure described above (12).

Salmon sperm DNA was obtained from Worthington Biochemicals Corp. and dissolved in SSC diluted 1:10 (0.1 SSC) to give a concentration of DNA of 1 mg/ml.

**Other materials.** *S*-adenosyl-L-[*methyl*-$^{14}$C]methionine (60.4 mCi/mmol; 0.02 mCi/ml) was obtained from New England Nuclear Corp. Unlabeled *S*-adenosyl-L-methionine was purified by the method of Shapiro and Hehninger (17). Aquasol was purchased from New England Nuclear Corp. Pancreatic deoxyribonuclease and pancreatic ribonuclease were obtained from Sigma Chemical Co. Phosphocellulose P11 was obtained from Whatman (Balsont, Ltd.); diethylaminoethyl-cellulose cellex D was obtained from Bio-Rad Laboratories.

**Transformation assay.** The preparation of *H. parainfluenzae* competent cells and transformation procedures used for *H. parainfluenzae* were those described by Nickel and Goodgal (14).

**Standard assay for restriction activity.** To test the effect of endonuclease R enzymes on the biological activity of DNA, DNA was diluted to 25 μg/ml in Tris-magnesium-mercaptoethanol buffer (6.6 mM each of Tris buffer, pH 7.4, $MgCl_2$, and 2-mercaptoethanol). To 0.1 ml of this material was added 5 to 10 μl of the enzyme from diethylaminoethyl-cellulose or phosphocellulose concentrate, and the mixture was incubated at 30 C for 60 min and then examined for transforming activity. Controls in these experiments consisted of DNA that was carried through the same regime without the addition of enzyme. The reduction of activity of 1,000-fold in 30 min for the streptomycin-resistant marker by *Hind III* or the dalacin marker by *Hind II* is equivalent to 0.02 units of enzyme of Smith and Wilcox (18).

**Standard assay for DNA methylase activity.** Unless otherwise indicated, reaction mixtures (125 μl) contained 50 mM potassium phosphate, pH 7.4; 1.2 mM 2-mercaptoethanol; 10 mM ethylenedinitrilotetraacetic acid, pH 7.0; 30 μg of DNA; 12 μM *S*-adenosyl-L-[$^{14}$C]methionine DNA; methylase (13 μg of protein).

After a 4-h incubation at 30 C (unless otherwise noted) the reaction was terminated by chilling, and 50 μg of salmon sperm DNA was added to each tube as a carrier. The DNA was precipitated by addition of 2 ml of cold 5% trichloroacetic acid. After 10 min on ice, the precipitates were collected on Whatman no. 3 paper filters in a multiple filtration manifold.

The filters were washed five times with cold 5% trichloroacetic acid and then measured for trichloroacetic acid-insoluble radioactivity in 5 ml of Aquasol in a Packard scintillation spectrometer. One picomole of $CH_3$ group is taken as 100 counts/min. Roy and Smith (15) defined 1 U of methylase as the incorporation of 1 pmol of $CH_3$ group into DNA in 1 h.

**Separation and purification of restriction endonucleases Hind II and Hind III.** Two different chromatographic steps were applied to separate the two

restriction activities. Unless otherwise indicated, all buffers used for the purification contained 0.5 mM ethylenedinitrilotetraacetic acid, and all steps were carried out at 4 C. The purification procedure for endonuclease R *H. influenzae* dIII is summarized in Table 1.

For a typical purification 14 liters of *H. influenzae* cells in brain heart infusion broth were grown at 37 C to optical density at 650 nm = 0.4, harvested by centrifugation in a Sharples centrifuge and stored frozen.

Thawed *H. influenzae* cells, 27 g, were washed once and resuspended in 20 ml of 0.05 M Tris, pH 7.4, 0.001 M glutathione. The cells were disrupted by treatment for 10 periods of 30 s at maximal output in an MSE ultrasonic disintegrator during which time the temperature of the suspension remained below 4 C. Cell debris was removed by centrifugation in a Spinco SW40.1 rotor at 36,000 rpm for 90 min at 4 C. The supernatant fluid (25 ml) was made 1 M by addition of solid NaCl and chromatographed on a Bio Gel A 0.5 M column (2.5 by 50 cm, Bio-Rad), equilibrated in 1 M NaCl, 0.02 M Tris, pH 7.4, 0.01 M mercaptoethanol buffer at a rate of flow of 50 ml/h. Two microliters of the 6-ml fractions were used in the assay to minimize the effects of high salt concentrations. The transformation tested the biological activity against *dal* and *str* marker of *H. parainfluenzae* DNA.

The restriction activity manifested against these markers was eluted as two separate components; the stronger component of activity inactivating the streptomycin resistance marker appeared at 0.36- to 0.4-column volumes, and the prominent activity against the dalacin resistance marker was found at 0.45 to 0.5-column volumes. Fractions containing activity against the streptomycin resistance marker were pooled (11 mg of protein per ml), dialyzed against 0.01 M $KPO_4$, pH 7.4, and applied at 9 ml/h to a phosphocellulose column (1.5 by 12 cm) previously equilibrated with the same buffer. The column was washed with 2 volumes of dialysis buffer and then a 250-ml linear gradient from 0.01 M to 0.5 M $KPO_4$, pH 7.4, was applied.

The activity against the streptomycin resistance marker was eluted between 0.1 to 0.15 M $KPO_4$ and was still contaminated with the activity against the dalacin resistance marker (eluted between 0.08 to 0.14 M $KPO_4$). Fractions active against the streptomycin resistance marker were pooled, dialyzed against 0.02 M Tris-hydrochloride, pH 8.3, 0.02 M KCl, 0.005 M mercaptoethanol, 0.005 M ethylenedinitrilotetraacetic acid, and applied to a column (1 by 8 cm) of diethylaminoethyl-cellulose equilibrated with the same buffer. The column was washed with 2 volumes of buffer and eluted using a linear gradient from 0.02 M to 0.5 M KCl (180 ml). The enzyme free of any detectable activity against the dalacin resistance marker was eluted between 0.04 to 0.07 M KCl and these fractions were pooled, concentrated fivefold with polyethyleneglycol, and then dialyzed against 0.02 M Tris, pH 7.4, 0.02 M KCl, 0.005 M mercaptoethanol and stored at 4 C.

The Bio Gel material that was eluted from 0.45- to 0.5-column volumes showed restriction activity against the dalacin resistance marker (Table 2). These fractions were dialyzed against 0.01 M $KPO_4$, pH 7.4, and applied at the rate of 18 ml/h to a phosphocellulose column (1.5 by 15 cm) equilibrated with the same buffer. The column was washed and volumes of dialysis buffer and developed with a linear 400-ml gradient from 0.01 to 0.5 M $KPO_4$, pH 7.4.

TABLE 1. *Purification of H. influenzae endonuclease R Hind III*

| Fraction | Vol (ml) | Protein (mg/ml) | *str* | Marker activity[a] *dal* |
|---|---|---|---|---|
| Crude extract | 25 | 68 | <0.01 | <0.01 |
| Bio Gel column (21-29) | 40 | 11 | <1 | <1 |
| Phosphocellulose | 20 | 0.13 | <0.01 | 17 |
| Diethylaminoethyl-cellulose concentrate | 2 | 0.08 | <0.01 | 100 |

[a] Percentage of activity remaining at 60 min.

TABLE 2. *Purification of H. influenzae endonculease R Hind II*

| Fraction | Vol (ml) | Protein (mg/ml) | *str* | Marker activity[a] (*dal*) |
|---|---|---|---|---|
| Crude | 25 | 68 | <0.01 | <0.01 |
| Bio Gel (30-43) | 90 | 3.4 | 1 | <0.1 |
| Phosphocellulose I | 24 | 0.35 | 7 | <0.01 |
| Phosphocellulose II concentrate | 1 | 0.08 | 83 | <0.01 |

[a] Percentage of activity remaining at 60 min.

The assay for endonuclease restriction enzyme revealed two activities between 0.05 to 0.14 M $KPO_4$. Maximal activity against the dalacin resistance marker was found at 0.06 M $KPO_4$ and against the streptomycin resistance marker at 0.1 M $KPO_4$.

The active fractions adjacent to the first component to be eluted were pooled, dialyzed against 0.01 M $KPO_4$, pH 6.3, and subsequently applied at 9 ml/h to a phosphocellulose column (1 by 8 cm) equilibrated with the same buffer. The column was washed, and then eluted with a 200-ml linear gradient, 0.05 to 1 M $KPO_4$, pH 6.3. Fractions of 2.8 ml were collected. The activity against the dalacin resistance marker was eluted at about 0.17 M $KPO_4$ and had little effect against the streptomycin resistance marker (83% of activity remaining).

**Purification of DNA modification methylases.** An attempt has been made to isolate the methylases that correspond to the two endonuclease restriction enzymes. Unless otherwise noted, all buffers contained 0.005 M 2-mercaptoethanol, 0.0005 M ethylenedinitrilotetraacetic acid, and 5% glycerol.

For the purification of the modification activity a crude extract was prepared from 20 g of frozen cells of *H. influenzae* strain Rd $com^{-10}$, following the procedure of Roy and Smith (15).

Crude extract (20 ml) containing 64 mg of protein per ml was treated overnight at 4 C with ribonuclease (heated at 80 C for 10 min) at a final concentration of 20 μg/ml and then brought to 1 M NaCl and applied to a Bio Gel A 0.5 M column (2.5 by 50 cm, Bio-Rad), equilibrated with 1 M NaCl, 0.02 M Tris, pH 7.4, 0.01 M mercaptoethanol. Elution was carried out with the same buffer at a flow rate of 50 ml/h. Fractions of 6 ml were collected and assayed for methylase activity using salmon sperm DNA as a substrate. Two components of methylating activity were found (see Fig. 1); the first activity was eluted from 0.23- to 0.46-column volumes and the second one was eluted from 0.5- to 0.75-column volumes.

Active fractions of the major component (fractions 33 to 51) were pooled (114 ml, 2.15 mg of protein per ml), dialyzed against 0.01 M $KPO_4$, pH 7.4, and then applied to a phosphocellulose Whatman PII column (1.5 by 15 cm) that had been equilibrated with the same buffer. The column was washed with 2 volumes of the equilibration buffer and eluted at 18 ml/h with a 400-ml linear gradient from 0.01 M to 0.5 M $KPO_4$, pH 7.4. Fractions of 4.5 ml were collected and assayed for DNA methylase activity on salmon sperm DNA and T7 DNA. Methylase activities were found in several components that were rechromatographed on a second phosphocellulose column as relatively pure individual components.

We are particularly interested in the activities IIa, IIb, and III that were eluted from the column at 0.07, 0.11, and 0.15 M $KPO_4$ (fractions 14, 20, and 26), respectively (Fig. 2). Three adjacent fractions of these activities were pooled, concentrated 10-fold with polyethyleneglycol, dialyzed against 0.01 M $KPO_4$, pH 7.0, diluted twofold with glycerol, and stored at −20 C.

## RESULTS

The restriction enzymes *Hind II* and *Hind III* were purified and shown to selectively inactivate the genetic markers of the DNA of *H. parainfluenzae*. The marker specificity of these

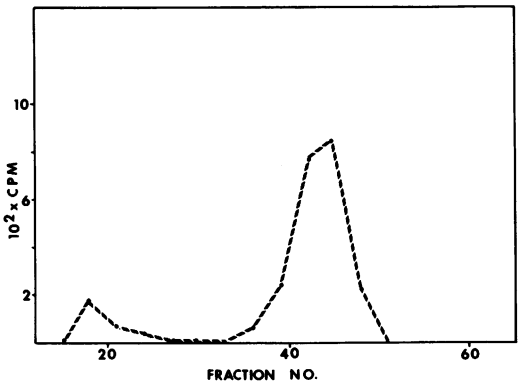


FIG. 1. *Bio Gel fractionation of methylase activities. Fractions demonstrating two major methylase activities separated according to size were assayed on salmon sperm DNA as described in Materials and Methods.*

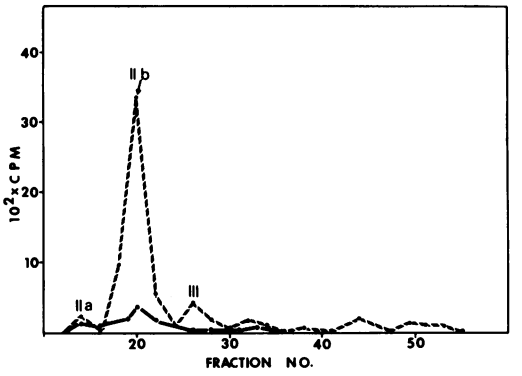


FIG. 2. *Fractionation of methylases from the major Bio Gel peak by phosphocellulose chromatography. Fractions were assayed for methylase activity on salmon sperm DNA (broken line, solid circle) and T7 DNA (solid line, star) as described in Materials and Methods.*

TABLE 3. *Activity of endonuclease (Endo) H. influenzae Endo R Hind II and III on H. parainfluenzae DNA*

| Enzyme | Marker activity of *H. parainfluenzae* DNA[a] | | | | |
|---|---|---|---|---|---|
| | *str* | *ery* | *nal* | *dal* | *nov* |
| Endo R *Hind II* | 53 | 40 | 40 | <0.01 | <0.01 |
| Endo R *Hind III* | <0.01 | <0.01 | <0.01 | 100 | 100 |

[a] Percentage of activity remaining at 60 min.

restriction endonucleases are given in Table 3. The *H. parainfluenzae* DNA treated with *Hind III* selectively inactivated the *str*, *ery*, and *nal* markers, but had no effect on the *nov* and *dal* markers that were found to be sensitive to the action of the second restriction enzyme *Hind II*. The three markers *str*, *ery*, and *nal* acid were mostly preserved after treatment with *Hind II*.

The endonuclease R preparations that Smith and co-workers (11, 18) isolated attacked T7 DNA introducing a limited number of double-strand breaks. To determine whether our two endonucleases correspond to those described by Smith and Wilcox (18), a preparation of T7 DNA was treated with the restriction enzymes and sedimented in neutral sucrose gradients. The results of this experiment are shown in Fig. 3. The *Hind III* enzyme that is active against the *str* marker had no effect on T7 DNA, whereas DNA, treated with the *Hind II*, active against the *dal* marker, was found to have its molecular weight reduced to $10^6$ as Smith and his group have previously shown.

**Substrate specificity of the DNA**

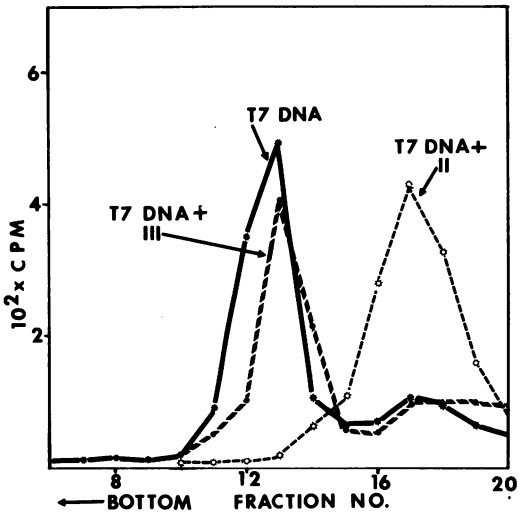


FIG. 3. *Sucrose density gradient sedimentation of T7 DNA after treatment with restriction enzymes. $^3$H-labeled T7 DNA (25 μg/ml) untreated or treated with 10 μl of an appropriate endonuclease R (from diethylaminoethyl-cellulose or phosphocellulose concentrate) was assayed using the conditions described in Materials and Methods. The reaction was stopped by the addition of 5 μl of 0.5 M ethylenedinitroltetraacetic acid (pH 7.4) and the samples were layered onto linear sucrose gradients (5 to 20%) with 0.1 M NaCl, 0.01 Tris-hydrochloride, pH 7.6, 0.001 M ethylenedinitrilotetraacetic acid. After centrifugation at 35,000 rpm for 3 h (15 C) in a Spinco SW50.1 rotor, the bottom of each tube was punctured and fractions (0.2 ml) were collected directly into scintillation vials containing 5 ml of Aquasol and were counted in a Packard scintillation spectrometer.*

**methylases.** A number of DNAs were used to assay for methylase activity. These data are shown in Table 4 and reveal that methylase IIa was active on salmon sperm DNA, showed some activity on *H. parainfluenzae* DNA and T7 DNA, and had no activity on *H. influenzae* DNA. The greatest activity on salmon sperm DNA was shown by methylase IIb (2,229 counts/min) which methylated all substrates tested except phage S2.25S DNA.

Methylase III, on the other hand, showed activity on *H. parainfluenzae* DNA but none for *H. influenzae* or T7 DNA. These data suggested that methylase III was the modification enzyme for the restriction enzyme *Hind III*, and that methylase IIa was associated with modification that prevented restriction by *Hind II*.

To prove these relationships we decided to methylate *H. parainfluenzae* DNA and to see whether or not we could obtain protection of this DNA from attack by the restriction enzymes *Hind II* and *Hind III*. The design of the experiment was as follows. (i) Donor *H. parainfluenzae* DNA was methylated with either methylase IIa or III and the DNA was then extracted with phenol to eliminate any residual enzyme; (ii) this DNA was treated with the restriction enzymes and then used to transform competent recipient *H. parainfluenzae* cells.

*Hind II* attacked the *dal* marker (Table 5); however, after treatment with methylase IIa most of this activity was prevented. The activity remaining after *Hind II* treatment was 40%. Omitting *S*-adenosylmethionine from the reaction resulted in complete loss of protection, i.e., inactivation to 0.01%.

TABLE 4. *Substrate specificity of DNA methylases*[a]

| Methylase | Counts/min | | | | |
|---|---|---|---|---|---|
| | Salmon sperm | DNA *H. parainfluenzae* | *H. influenzae* Rd | T7 B | S2.25S |
| IIa | 745 | 68 | 0 | 47 | 0 |
| IIb | 2,229 | 32 | 147 | 713 | 0 |
| III | 838 | 474 | 0 | 0 | 0 |

[a] Reactions were carried out as described in Materials and Methods using a concentrated enzyme stored frozen at 20 C in 50% glycerol.

TABLE 5. *Protection of transforming activity of H. parainfluenzae DNA from inactivation by H. influenzae endonuclease (Endo) R Hind II*[a]

| Enzyme treatment | % Activity of marker remaining at 60 min | |
|---|---|---|
| | *str* | *dal* |
| Endo R | 53 | <0.01 |
| No Endo R | 100 | 100 |
| Methylase IIa (SAM+) + Endo R | 53 | 40 |
| Methylase IIa (SAM−) + Endo R | (−) | <0.01 |
| Methylase III (SAM+) + Endo R | 53 | 0.2 |

[a] Modification reaction: each reaction mixture (250 μl) contained: 100 mM potassium phosphate, pH 7.4, 2.4 mM 2-mercaptoethanol, 20 mM ethylenedinitrilotetraacetic acid, pH 7.0, 240 μM *S*-adenosylmethionine (SAM), 60 μg of DNA, DNA methylase (26 μg of protein). After 18 h of incubation at 30 C each sample was extracted with an equal volume of cold phenol. The aqueous layer containing the DNA was dialyzed extensively against 0.05 M $KPO_4$, pH 7.4. Restriction challenge: the treated DNA was diluted 10-fold in Tris-magnesium-mercaptoethanol buffer (6.6 mM each of Tris buffer, pH 7.4, $MgCl_2$, and mercaptoethanol). To 0.1 ml of this material was added 10 μl of purified restriction enzyme and then it was incubated at 30 C. Samples were removed after 60 min and examined for transforming activity.

TABLE 6. *Protection of transforming activity of H. parainfluenzae DNA from inactivation by H. influenzae Endo R Hind III*

| Enzyme treatment | % Activity of marker remaining at 60 min | |
|---|---|---|
| | *str* | *dal* |
| Endo R | <0.01 | 100 |
| No Endo R | 100 | 100 |
| Methylase III (SAM+) + Endo R | 60 | 100 |
| Methylase III (SAM−) + Endo R | 1.0 | 100 |
| Methylase IIa (SAM+) + Endo R | 0.6 | 100 |

In Table 6 it can be seen that *Hind III* affected only the *str* marker (<0.01% activity remaining) and that methylase III prevented most of the activity of this enzyme related to the *str* marker (60% activity remaining). In this experiment in the absence of added *S*-adenosylmethionine methylase III gave some protection to the *str* marker. No protection was afforded by methylase IIa to *H. parainfluenzae* DNA subsequently treated with *Hind III*, and methylase III provided little protection toward *Hind II* (Tables 5, 6).

## DISCUSSION

Endonuclease R of *H. influenzae* and other restriction enzymes belong to a class of proteins that act at a limited number of specific sites within the DNA duplex. *H. influenzae* restriction endonuclease reported by Smith and Wilcox (18) has been shown to make approximately one break per 1,000 base pairs.

Kelly and Smith (11) have determined the base sequence of the sites that are recognized by this restriction enzyme. The sequence is symmetric and contains the following nucleotides: 5′- - -GpTpPy ↓ pPupApC- - -3′ and 3′- - -CpApPup ↓ PypTpG- - -5′, where the arrows indicate the sites of phosphodiester bond hydrolysis.

Recently Roy and Smith (15) have purified four DNA adenine methylases from *H. influenzae* Rd and shown that methylase II is the modification enzyme whose specificity corresponds to endonuclease R of Smith and Wilcox. Furthermore, the sequence of the nucleotides methylated by methylase II has been determined (16) and found to be consistent with the recognition site determined by Kelly and Smith (11) for the *H. influenzae* restriction enzyme, endonuclease R. The partial recognition site for methylase II is (5′) pPupmApC, and it is probable that the presence of methyl groups confer protection against cleavage by steric hindrance to the binding of endonuclease *Hind II*.

Thus, one biological role of methylated bases in DNA is that of protecting certain regions of DNA from endonucleolytic attack. However, the methylation of bacterial and phage DNA involved in host-controlled modification represents only a small part of the total number of methylated bases in a given genome, and the biological role of the majority of methylated bases in bacterial and phage DNA remains unknown.

The endonuclease R preparation of Smith and Wilcox is now known to have consisted of two endonucleases, *Hind II* and *Hind III* (18). The activity against T7 DNA was conferred by *Hind II* (15).

Goodgal and Gromkova (7) have shown that upon endonuclease R treatment *H. parainfluenzae* DNA had been cleaved to pieces with an average size of $10^6$ daltons and these segments were no longer capable of transforming the *str*, *ery*, and *dal* markers. Sucrose gradient fractionation of DNA after sonic oscillation confirms the fact that small segments of *H. parainfluenzae* DNA in general do not carry the sites for homospecific recombination. These observations encouraged us to purify the specific modification enzymes from *H. influenzae* to provide *H. parainfluenzae* DNA with a sufficient degree of modification to protect it against either one or both *H. influenzae* restriction endonucleases.

Methylase IIa (which methylates T7 DNA) corresponds to the methylase II described by Roy and Smith (15). This methylase was able to protect 40% of the *dal* marker of *H. parainfluenzae* DNA from restriction by *Hind II* at a level of methylation of approximately 30 methyl groups per molecule.

On the other hand methylase III did not methylate T7 DNA, and was found to protect *H. parainfluenzae* DNA from cleavage by restriction endonuclease *Hind III* with about 60% of the original $str^r$ activity restored. This result may be due to the introduction of a greater number of methylated sites in *H. parainfluenzae* DNA.

Methylase IIb appears to be a different enzyme, having a unique substrate specificity. This preparation of enzyme revealed a rather complex methylation pattern and showed activity on *H. influenzae*, phage T7 B, and *H. parainfluenzae* DNAs, but not on phage S2.25S DNA.

From its elution characteristics on phosphocellulose methylase IIb might have been the methylase described by Roy and Smith (15) as corresponding in specificity to the type I restriction system of Glover and Piekarowicz (4). However, there was no methylation of phage

S2.25S DNA. The absence of activity of this enzyme towards DNA from phage grown on a phenotypically $r^- m^-$ recipient of *H. influenzae* Rd suggests that this methylase has a different specificity, and presumably corresponds to another restriction endonuclease that we have recently isolated. The characteristics of these enzymes will be described later.

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## LITERATURE CITED

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