Biological Properties of a *Haemophilus influenzae* Restriction Enzyme, *Hind I*

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A type I restriction enzyme from *Haemophilus influenzae, Hind I*, which requires adenosine 5'-triphosphate and 5-adenosyl methionine, was studied for its activity on transfecting and transforming deoxyribonucleic acid (DNA). The enzyme reduced the size of unmodified bacteriophage S2 DNA from $37 \times 10^9$ daltons to approximately $10 \times 10^8$ daltons, but did not affect modified S2 DNA. Unmodified transforming DNA was attacked in vitro by *Hind* I; however, relatively low levels of inactivation were obtained for single markers, and linked transformants were inactivated as a function of the distance between markers. In contrast, unmodified bacterial DNA was not inactivated in vivo for either single or linked markers by the *Hind I* restriction system, probably because the segments generated by *Hind I* were still capable of being integrated in vivo. The lack of preferential inactivation of markers by the enzyme suggests that it makes random breaks in the DNA.

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**MATERIALS AND METHODS**

Bacterial strains used. *H. influenzae* strain Rd was originally obtained from Alexander and Leidy (1). For isolation of the phage restriction enzyme *Hind I*, a restrictive segregant from a competence deficient strain Rd com-16 was used. The strain was shown to be deficient in an exonuclease described by Gunther and Goodgal (13). In transfection and transformation experiments restrictive (2R) and nonrestrictive (19S or 25S) segregants from strain Rd were used (10). *H. influenzae* Reid and *H. parainfluenzae* were obtained from Grace Leidy.

Bacteriophages. *H. influenzae* bacteriophage S2 was obtained from a *Haemophilus* species isolated from a patient of the University of Pennsylvania Hospital (S. H. Goodgal, Fed. Proc. 23:518, 1964).

Media. For the growth of *H. influenzae* and *H. influenzae* Reid, brain heart infusion medium (Difco) was supplemented with 10 μg of hemin (Eastman Kodak) per ml and 2 μg of nicotinamide adenine dinucleotide (Nutritional Biochemicals Corp.) per ml. *H. parainfluenzae* was grown in brain heart infusion medium obtained from Baltimore Biological Laboratories and 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; streptovaracin (dalacin), obtained from the Upjohn Co., was dissolved in 50% ethanol and was used at a final concentration of 8 μg/ml. Novobiocin (Upjohn) and erythromycin (Eli Lilly) dissolved in water were both used at a final concentration of 10 μg/ml.

Preparation of DNA. Bacterial and prophage DNAs were obtained by the procedures of Berns and Thomas (2) as modified by Michalka and Goodgal.
(19). Bacteriophage S2 DNA was prepared by growing lysogenic cells at 37°C to a concentration of $5 \times 10^8$ per ml and then adding mitomycin C to a final concentration of 0.05 $\mu$g/ml. Incubation was continued with vigorous aeration for 130 to 150 min, at which time the turbidity had dropped 50% or more. Pancreatic deoxyribonuclease was added at $1 \mu$g/ml and ribonuclease was added at $20 \mu$g/ml, and incubation was continued for 30 min at 37°C. The cell debris was removed by centrifugation at 5,000 rpm in Sorvall 2B centrifuge, and the phage in the supernatant fluid was precipitated with 5% polyethylene glycol by the procedure of Yamamoto et al. (24). The pellet was suspended in MIC media (14), and the phage DNA was extracted by adding an equal volume of aqueous neutralized phenol (pH 7). In general, three 10-min extractions were employed.

Preparation of $^3$H-labeled bacteriophage DNA. $^3$H-labeled DNA was prepared by the addition of 5 $\mu$Ci of [3H]thymidine per ml and 2 mg of inosine per ml to the growing culture when the cells reached a density of $5 \times 10^8$ per ml.

Transformation and transfection assays. The transformation procedure for $H. influenzae$ has been described (9). Competent cells were prepared by the method of Goodgal and Herriott as modified by Cameron (9). The same procedure was used for $H. influenzae$ Reid transformation. Details for the assay are described in a previous paper (7). The preparation of $H. parainfluenzae$ competent cells and the transformation procedure used for $H. parainfluenzae$ were those described by Nickel and Goodgal (20).

Purification of the Hind I enzyme. A slight modification of the procedure described by Smith and Wilcox (23) for purification of type II endo R was used for the preparation of Hind I restriction enzyme. The Hind I restriction activity is found in the 0 to 45% (NH$_4$)$_2$SO$_4$ fraction, whereas the type II endo R activity appeared in the 45 to 70% (NH$_4$)$_2$SO$_4$ fraction. Upon phosphocellulose chromatography, the bulk of Hind I restriction activity eluted with 0.3 M KCl (10). The enzyme was further purified by sedimentation in a 10 to 25% glycerol gradient (6 h at 50,000 rpm in a Spinco SW65 rotor). The 0.3 M KCl fractions contained 500 to 1,500 units of enzyme/mg of protein.

Enzyme assay. Bacterial or bacteriophage DNA was diluted to 25 $\mu$g/ml in TMS buffer [tris(hydroxymethyl)aminomethane buffer, pH 7.4, MgCl$_2$, and mercaptoethanol, each at 6.6 mM]. The assay was performed in the presence and absence of $H. influenzae$ 2R (0.2 $\mu$mol/ml) and SAM (0.02 $\mu$mol/ml). To 0.1 ml of DNA 5 $\mu$l of purified enzyme was added, and the mixture was incubated at 30°C. Samples were removed after various times of incubation and examined for transforming or transfecting activity. Controls in these experiments consisted of untreated DNA. One unit of enzyme is defined as that amount of enzyme that reduces transfecting activity by 50% in 30 min at 30°C in the standard assay system.

Sucrose density gradient centrifugation. Portions (0.1 ml) of unmodified and modified $^3$H-labeled bacteriophage S2 DNA (25 $\mu$g/ml) were treated with 20 $\mu$l of purified Hind I restriction enzyme (20 units) in the presence and absence of ATP and SAM. After 120 min, 20 $\mu$l of the incubation mixture was layered on top of a 5-ml linear 5 to 20% sucrose gradient containing 0.3 M NaCl. T7 DNA labeled with [14C]thymidine and T4 DNA labeled with $^{32}$P were used as sedimentation velocity markers. The size of these DNAs were taken to be $130 \times 10^6$ daltons for T4 and $26 \times 10^6$ daltons for T7. Samples were then centrifuged at 45,000 rpm for 2.5 h at 18°C in a Spinco SW65 rotor. Twenty-nine fractions were collected and analyzed for the distribution of radioactivity.

Viscometry. The viscometric assay for endonuclease activity has been described (11, 23). The reaction mixture contained 250 enzyme units in 2.5 ml of unmodified DNA at 50 $\mu$g/ml. As a control the unmodified DNA was treated with the $H. parainfluenzae$ endonuclease R. (11).

**RESULTS**

In vitro effect of Hind I on biologically active DNA. The restriction enzyme Hind I has been shown to have a specific effect on unmodified transfecting DNA (10). To determine the effect of Hind I on the activity of transforming DNA, the enzyme was tested on different genetic markers of three Haemophilus species ($H. influenzae$, $H. parainfluenzae$, and $H. influenzae$ Reid). The results (Table 1) show that unmodified homologous and heterologous bac-

| DNA species            | % Activity remaining at 60 min$^*$ |
|------------------------|-----------------------------------|
| $H. influenzae$ 2R (modified) | 100 100 100 100 |
| $H. influenzae$ 19S (unmodified) | 30 45 40 35 |
| $H. influenzae$ Reid    | 49 62 42 54 |
| $H. parainfluenzae$     | 50 80 65 NT$^c$ |

$^*$ The reaction mixture contained 0.1 ml of DNA at 25 $\mu$g/ml, 5 $\mu$l of ATP (0.2 $\mu$mol/ml), 5 $\mu$l of SAM (0.02 $\mu$mol/ml), and 5 $\mu$l of enzyme (5 units). After 60 min of incubation at 30°C, 50 $\mu$l of the mixture was added to the transformation assay tube consisting of 2.8 ml of brain heart infusion broth and 0.2 ml of competent cells. See Materials and Methods and reference 7 for further details concerning the assay.

$^c$ The enzyme Hind I preparation contained 1 mg of protein/ml and approximately 1,000 units of enzyme/ml. The same preparation was used in the experiments presented in Tables 2, 3, and 4.

$^*$ Percent activity remaining relative to activity after treatment in the absence of ATP and SAM.

NT, Not tested.
terial DNAs were only slightly affected by the Hind I restriction enzyme, whereas modified DNA was not affected. No loss of transforming activity was observed in the absence of ATP and SAM, indicating that the inactivation was due to a type I restriction enzyme. Continued incubation for periods of up to 3 h as well as increasing the amount of enzymes did not lead to any further inactivation of the transforming DNA. Furthermore, the inactivation of the markers tested occurred to approximately the same level. In the case of inactivation of heterologous DNA by type II restriction enzymes (7, 8), it was found that some markers were preferentially inactivated, and the extent of inactivation was much greater than that produced by Hind I.

When the same Hind I enzyme preparation was tested for inactivation of bacteriophage DNA, it was found that transfection is much more sensitive to the enzyme than transformation (Table 2).

Activity of Hind I enzyme on linked markers in bacterial transformation. Since Hind I enzyme was not very efficient in inactivation of single bacterial markers, we examined the effect of this enzyme on linked bacterial markers. It is known that the number of linked transformants is dependent upon the size of the DNA segment. We anticipated that if the enzyme made only a few breaks in the DNA, the efficiency of linked transformants would be affected more strongly than that of single transformants. Unmodified and modified H. influenzae DNAs were used as donors, and linked, drug-resistant transformants were examined (str nov, str dal), as well as the corresponding single markers (str, nov, dal).

### Table 2. Transfecting activity of phage and prophage S2 DNA after treatment with Hind I enzyme

| DNA                  | % Activity remaining at 60 min<sup>a</sup> |
|----------------------|------------------------------------------|
|                      | Without ATP and SAM | With ATP and SAM |
| Phage S2·19S (unmodified) | 100                | 1                |
| Prophage 19S (S2) (unmodified) | 100                | 2                |
| Phage S2·2R (modified)      | 100                | 90               |
| Prophage 2R (S2) (modified) | 100                | 95               |

<sup>a</sup> See footnote a, Table 1 for procedures used for inactivation of DNA.

The results of treatment of unmodified DNA are presented in Table 3, and the results using modified DNA are presented in Table 4. Modified bacterial DNA is almost completely resistant to the action of Hind I enzyme. When unmodified H. influenzae DNA was tested, the transforming efficiency of single markers was slightly reduced (Table 3). However, the rate of inactivation of the double transformants was considerably higher and depended upon the distance between the markers. The str nov pair with a linkage distance that is approximately $10 \times 10^6$ daltons was less affected than the str dal markers, which are about $40 \times 10^6$ daltons apart.

**Effect of Hind I enzyme on DNA degradation: viscometry.** To obtain a crude estimate of the digestion of DNA, an Ostwald viscometer was used to measure the specific viscosity of unmodified H. influenzae DNA as a function of treatment with Hind I (Fig. 1). This has previously been shown that type II deoxyribonuclease from H. influenzae and H. parainfluen-

### Table 3. Effect of Hind I enzyme on unmodified transforming DNA

| Marker | Without ATP and SAM | With ATP and SAM |
|--------|---------------------|------------------|
|        | -E<sup>b</sup>       | +E               |
| str    | 2,500               | 3,600            |
| nov    | 3,200               | 2,650            |
| dal    | 4,200               | 4,500            |
| str nov| 1,152              | 1,040            |
| str dal| 106                | 151              |

<sup>b</sup> See footnote a, Table 1 for procedures used for inactivation of DNA.

<sup>c</sup> Sixteen of these are calculated to be due to random doubles (5).

### Table 4. Effect of Hind I enzyme on modified transforming DNA

| Marker | Without ATP and SAM | With ATP and SAM |
|--------|---------------------|------------------|
|        | -E                  | +E               |
| str    | 3,950               | 4,500            |
| nov    | 3,330               | 3,180            |
| dal    | 3,840               | 2,980            |
| str nov| 1,000              | 690              |
| str dal| 104                | 81               |

<sup>a</sup> See footnote a, Table 1 for procedures used for inactivation of DNA.
enzae reduced the specific viscosity of foreign DNAs (11, 23) as measured in an Ostwald viscometer and that the size of fragments produced was of the order of $10^6$ daltons. In contrast, Hind I did not produce any appreciable drop in the viscosity of the unmodified DNAs tested. It should be noted that the viscometer is not sufficiently sensitive to detect viscosity differences when the sizes of the DNAs are greater than $8 \times 10^6$ daltons. These experiments demonstrate only that Hind I does not produce many breaks in unmodified DNA.

Sucrose gradient sedimentation. Degradation of DNA by the Hind I enzyme was further studied by sucrose gradient sedimentation. $^3$H-labeled modified and unmodified bacteriophage S2 DNAs were treated with Hind I enzyme in the presence and absence of ATP and SAM. The results (Fig. 2) show that there is a definite shift only in the case of unmodified DNA treated with Hind I enzyme in the presence of enzyme cofactors. In the absence of ATP and SAM no shift occurred. The position of modified S2·2R DNA treated with Hind I in the presence of ATP and SAM coincided with that of the untreated DNAs, except for a small shoulder that can be accounted for by the presence of a small amount of unmodified DNA in the preparation of S2·2R DNA (see references 6 and 10 for an explanation of this phenomenon). The fact that the distance between the sedimentation peaks of treated DNA versus DNA treated in the absence of ATP and SAM was not very great suggested that the restriction enzyme Hind I made only a few breaks. The size of the S2 DNA in the original preparation was found to be greater than the T7 DNA standard that had a size of $26 \times 10^6$ daltons and agrees with the size of $37 \times 10^6$ daltons determined by J. W. Bendler (Ph.D. thesis, John Hopkins Univ., Baltimore, 1968). The size of the fragments produced by Hind I was approximately $10 \times 10^6$ daltons, as determined by the procedure of Hershey et al. (15). This size represents the limit of digestion of unmodified H. influenzae DNA by Hind I, since extended incubation for 24 h or increasing the enzyme concentration fivefold produced DNA fragments with the same sedimentation velocity as that shown in Fig. 2 for the action of the enzyme on unmodified DNA.

Role of the Hind I enzyme on exclusion of DNA in vivo. To study the effect of the Hind I restriction system in vivo, the efficiencies of transfection and transformation were compared in restrictive and nonrestrictive recipients. The results of these experiments (Tables 5 and 6) demonstrate that the efficiency of transfection and the plating efficiency of phage are functions of DNA modification and the presence of restriction in the recipient (Table 5). For unmodified DNA, transfecting activity and the plating efficiency of bacteriophage S2 are reduced in the restrictive recipient (Table 5). Modified phage or DNA from this phage show the same relative activity in restrictive and nonrestrictive recipients. In transformation the efficiencies of single and linked markers were examined, but no reduction in the number of single and double transformants was observed in vivo when the restrictive host was used as a recipient and unmodified DNA as a donor (Table 6). These same markers were sensitive when treated with Hind I enzyme in vitro (Table 3). One may conclude therefore that the Hind I restriction enzyme that can attack transforming DNA in vitro is somehow incapable of inactivating this DNA in vivo.

DISCUSSION

Previous studies on the biological effects of type II endo R enzymes isolated from different Haemophilus species (H. influenzae, H. parainfluenzae, and H. aegyptius) revealed similarities in their biological effects, suggesting similar modes of action (8). Type II endodeoxyribonucleases demonstrate very strong in vitro inactivation of heterologous transforming DNA

**Fig. 1. Ostwald viscometry of unmodified H. influenzae DNA treated with H. parainfluenzae endonuclease (Hpa I) or Hind I. See Materials and Methods for experimental details. Symbols: O, H. parainfluenzae enzyme; ●, Hind I enzyme.**
with preferential survival of some markers (7, 8). By the use of physical and biological methods it has been demonstrated that these enzymes produce specific breaks in DNA (4, 5, 7, 17). This property of type II restriction enzymes has been used to generate specific DNA fragments for studying DNA structure and function (4, 5). Furthermore, it has been shown that these enzymes break DNA at recognition sites that are also sites for methylation (17, 22). Type I restriction enzymes appear to have a different mode of action. Horiuchi and Zinder demonstrated that the sites of breakage of Escherichia coli B restriction enzyme are different from the recognition sites (16). The facts that Hind I enzyme does not show preferential marker inactivation and all markers tested are affected almost to the same extent suggest that this enzyme makes random breaks. The major similarity in the biological effect of Hind I and type II endodeoxyribonuclease is that they do not attack specifically modified DNA (7, 8, 11).

Compared to the type II endonucleases, the number of breaks made by Hind I is limited.
Whereas the type II endonuclease from *H. parainfluenzae* reduces the size of *H. influenzae* DNA to approximately 1 × 10^6 daltons, unmodified S2 DNA or unmodified *H. influenzae* DNA (experiments not shown) are reduced to a size of approximately 10 × 10^6 daltons by Hind I. If the size of the segment is rendered smaller than the intact size required for biological function, it can be considered inactivated. In the case of S2 phage DNA, the effect of Hind I is to produce fragments below the minimal size required for transfection. For transforming DNA, single markers can be transformed on segments of 10 × 10^6 daltons; however, linked markers greater than 10 × 10^6 daltons apart would become unlinked and behave as separate segments after Hind I treatment. As shown above, the markers that are farther apart are separated more readily than closely linked markers. All of the properties discussed above have to do with the in vitro effects of Hind I. If one considers the actions of the enzymes in vivo, one observes that cells carrying Hind I restrict unmodified phage DNA but have no effect on unmodified transforming DNA. Although this observation may be explained by the presence of a larger number of recognition sites for the enzyme on the bacteriophage DNA, it is not likely, since no appreciable drop of specific viscosity of bacteriophage DNA was observed after treatment with the enzyme, and the size of the fragments of unmodified phage and bacterial DNAs were approximately the same. It is more likely that the greater inactivation of transfecting activity is due to the requirement for large intact phage DNA for transfection. Any reduction in size of the DNA will lead to inactivation. Unlike Hind I enzyme, type II restriction enzymes are not efficient in inactivation of unmodified bacteriophage S2 and bacteriophage S2 DNA in vivo (12, 21). If one assumes that Hind I reduces unmodified transforming DNA to an average size of 10 × 10^6 daltons, then one could postulate that there would be little inactivation in vivo, since segments this size can be integrated with a high efficiency.

A similar relationship between in vitro and in vivo results was observed in studying the effect of *H. influenzae* type II endonucleases in heterospecific transformation (8). It was found that *H. aegyptius* and *H. influenzae* Reid DNAs were sensitive to *H. influenzae* type II endonucleases; however, these DNAs were very efficient donors in interspecific transformation when *H. influenzae* was used as a recipient. These data show that the ability of these enzymes to attack DNA is in some way controlled in vivo. It is not clear that the ability of the type II and Hind I enzymes to attack DNA is controlled by the same mechanism.

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