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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 37×10^6 daltons to approximately 10×10^6 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.

A number of restriction enzymes from *Haemophilus* species have been studied for their ability to make specific breaks in deoxyribonucleic acid (DNA) (4, 5) as well as for their action on biologically active DNA (7, 8, 11). These studies have led us to attempt to correlate the in vitro effect of restriction enzymes with their possible role in vivo (9). Most of the restriction enzymes from *Haemophilus* belong to type II restriction enzymes according to the classification system proposed by Boyer (3). These enzymes require only Mg^{2+} for their action in contrast to type I enzymes that require Mg^{2+}, adenosine 5'-triphosphate (ATP), and S-adenosyl methionine (SAM) (18). Recently we have purified a restriction enzyme from *H. influenzae, Hind* I, involved in the restriction of *H. influenzae* bacteriophage S2 and HP1c1 and have shown that it is a type I enzyme (10).

In this paper we present data on the effect of *Hind* I restriction on transfecting and transforming activities of different DNAs. It was found that the *Hind* I enzyme in vitro inactivates transfecting and transforming activity of unmodified bacteriophage, prophage, and bacterial DNAs. The specific activity for different markers and the extent of inactivation by *Hind* I restriction enzyme were different from *H. influenzae* type II restriction enzymes. In addition, it may be concluded that the *Hind* I enzyme plays a role in exclusion of bacteriophage S2 in vivo but is not efficient in vivo in exclusion of unmodified bacterial DNA.

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^{-10} 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. 23:318, 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 5×10^8 per ml and then adding mitomycin C to a final concentration of 0.05 μg/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 μg/ml and ribonuclease was added at 20 μg/ml, and incubation was continued for 30 min at 37°C. The cell debris was removed by centrifugation at 5,000 rpm in a 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 μCi of [^{3}H]thymidine per ml and 2 mg of inosine per ml to the growing culture when the cells reached a concentration of 5×10^8 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 was found in the 0 to 45% $(NH_4)_2SO_4$ fraction, whereas the type II endo R activity appeared in the 45 to 70% $(NH_4)_2SO_4$ 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 μg/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 ATP (0.2 μmol/ml) and SAM (0.02 μmol/ml). To 0.1 ml of DNA 5 μl 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 μg/ml) were treated with 20 μl of purified *Hind* I restriction enzyme (20 units) in the presence and absence of ATP and SAM. After 120 min, 20 μl 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 [^{14}C]thymidine and T4 DNA labeled with ^{32}P were used as sedimentation velocity markers. The size of these DNAs were taken to be 130×10^6 daltons for T4 and 26×10^6 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 μg/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-

TABLE 1. *Inactivation of transforming activity of DNA species after treatment for 60 min with Hind I enzyme in the presence of ATP and SAM*[a]

DNA species	% Activity remaining at 60 min[b]			
	str	*nov*	*ery*	*dal*
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]

[a] The reaction mixture contained 0.1 ml of DNA at 25 μg/ml, 5 μl of ATP (0.2 μmol/ml), 5 μl of SAM (0.02 μmol/ml), and 5 μl of enzyme (5 units). After 60 min of incubation at 30°C, 50 μl 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. 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.

[b] Percent activity remaining relative to activity after treatment in the absence of ATP and SAM. There was no nonspecific endonuclease activity in this enzyme preparation. Untreated DNAs were 100% active.

[c] 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^r nov^r, str^r dal^r), as well as the corresponding single markers (str^r, nov^r, dal^r).

TABLE 2. *Transfecting activity of phage and prophage S2 DNA after treatment with Hind I enzyme*[a]

DNA	% Activity remaining at 60 min[b]	
	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

[a] See footnote *a*, Table 1 for procedures used for inactivation of DNA.

[b] Efficiency of transfection of 100 represents 2×10^5 plaque-forming units per ml or approximately 10^9 phage equivalents 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 considerable higher and depended upon the distance between the markers. The str^r nov^r pair with a linkage distance that is approximately 10×10^6 daltons was less affected than the str^r dal^r markers, which are about 40×10^6 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). It has previously been shown that type II deoxyriboendonuclease from *H. influenzae* and *H. parainflu-*

TABLE 3. *Effect of Hind I enzyme on unmodified transforming DNA*[a]

Marker	No. of transformants			
	Without ATP and SAM		With ATP and SAM	
	−E[b]	+E[b]	−E	+E
str	2,500	3,600	3,600	1,700
nov	3,200	2,650	3,100	1,900
dal	4,200	4,500	4,100	2,900
str nov	1,152	1,040	1,252	216
str dal	106	151	129	25[c]

[a] See footnote *a*, Table 1 for procedures used for inactivation of DNA.

[b] −E, Untreated with enzyme; +E, treated with enzyme.

[c] Sixteen of these are calculated to be due to random doubles (5).

TABLE 4. *Effect of Hind I enzyme on modified transforming DNA*[a]

Marker	No. of transformants			
	Without ATP and SAM		With ATP and SAM	
	−E	+E	−E	+E
str	3,950	4,500	3,810	3,600
nov	3,330	3,180	3,380	3,000
dal	3,840	2,980	4,590	2,980
str nov	1,000	690	939	810
str dal	104	81	109	64

[a] See footnote *a*, Table 1 for procedures used for inactivation of DNA.

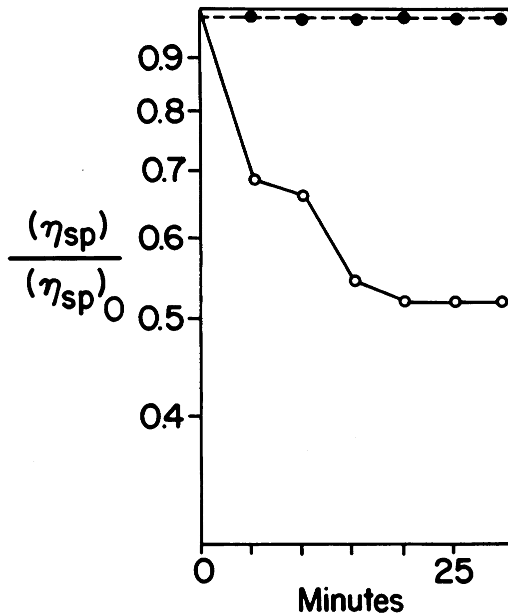

FIG. 1. *Ostwald viscometry of unmodified H. influenzae DNA treated with H. parainfluenzae endonuclease (Hpa I) or Hind I. See Materials and Methods for experimental details. Symbols:* ○, *H. parainfluenzae enzyme;* ●, *Hind I enzyme.*

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 × 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 × 10^6 daltons and agrees with the size of 37 × 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 × 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

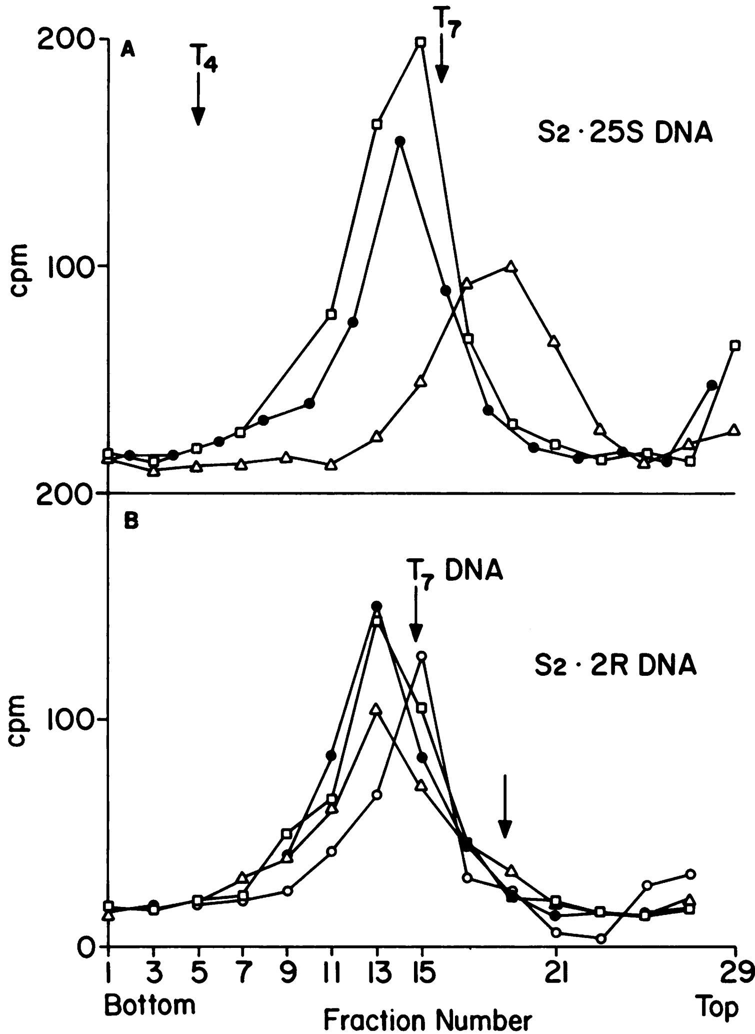

FIG. 2. *Neutral sucrose sedimentation gradient of unmodified (S2 ·25S) and modified (S2 ·2R) ^{3}H-labeled phage DNAs treated with Hind I in the presence and absence of ATP and SAM. See Materials and Methods for experimental details. Symbols: ●, control (no enzyme treatment); □, treatment with enzyme in the absence of ATP and SAM; △, treatment with enzyme in the presence of ATP and SAM; ○, T7 DNA. (A). Arrows indicate the positions of reference ^{32}P-labeled T4 DNA and ^{14}C-labeled T7 DNA; (B) arrow indicates the position of unmodified H. influenzae DNA treated with Hind I.*

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.

TABLE 5. *Plating and transfecting efficiency of phage S2 and phage S2 DNA in nonrestrictive and restrictive recipients*

Phage or DNA	Efficiency of[a] phage plating		Efficiency of[b] transfection	
	19S	2R	19S	2R
S2·19S (unmodified)	1	5×10^{-2}	1	10^{-1}
S2·2R (modified)	1	1	1	1

[a] An efficiency of plating of 1 is equal to 2×10^{10} plaque-forming units per ml.
[b] An efficiency of transfection of 1 is equal to 10^5 plaque-forming units per ml.

TABLE 6. *Transforming efficiency of unmodified H. influenzae DNA*[a] *in restrictive and nonrestrictive recipients*

Marker	No. of transformants with recipient:	
	Restrictive	Nonrestrictive
str	643	482
nov	457	472
dal	623	656
str nov	148	136
str dal	34	20

[a] DNA was used at a concentration of 25 μg/ml.

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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