Action of Restriction Endonucleases on Transforming DNA of *Haemophilus influenzae*

KENNETH L. BEATTIE,* ADIL E. WAKIL, AND PAUL H. DRIGGERS

Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030

Received 9 November 1981/Accepted 15 June 1982

Cleavage of DNA from *Haemophilus influenzae* with restriction endonucleases caused inactivation of transforming ability to an extent that depended on the genetic marker and the enzyme. The rate of inactivation, but not the final level of survival, depended on the concentration of enzyme in the restriction digest. In general, the greatest extent of inactivation of transforming activity was obtained with endonucleases that are known to produce the shortest fragments. We electrophoresed restriction digests of *H. influenzae* DNA in agarose gels and assayed transforming activity of DNA extracted from gel slices. In this way, we determined the lengths of restriction fragments that contain genetic markers of *H. influenzae*. For the marker that we studied most thoroughly (*nov*), the shortest restriction fragment that possessed detectable transforming activity was a 0.9-kilobase pair fragment produced by endonuclease R · *Pst*I. The shortest marker-bearing restriction fragment that retained substantial transforming activity (50% of value for undigested DNA) was a 2.1-kilobase pair *Eco*RI fragment bearing the *kan* marker. Among marker-bearing restriction fragments 1 to 4 kilobase pairs in length, survival of transforming activity varied 10,000-fold. We relate these observations to the recent finding by Sisco and Smith (Proc. Natl. Acad. Sci. U.S.A. 76:972-976, 1979) that efficient entry of DNA into competent *H. influenzae* cells appears to require the presence of a recognition sequence that is scattered throughout the *Haemophilus* genome in many more copies than in unrelated genomes.

As part of a long-term investigation of the enzymology of mutagenesis, our laboratory seeks to develop a system for in vitro mutagenesis, utilizing small, well-defined DNA molecules bearing useful genetic markers, which would be subject to efficient in vitro replication and repair. Toward this aim, we decided to construct recombinant plasmid DNAs containing various genetic markers from the highly transformable species *Haemophilus influenzae*. Since our assay of in vitro mutagenesis is to be a genetic one (the *H. influenzae* transformation system) and because the *H. influenzae* DNA to be inserted into the plasmids consists of discrete restriction fragments bearing genetic markers, it is essential that these fragments retain transforming activity. A recently discovered peculiarity of the *H. influenzae* transformation system suggested to us that this criterion might be difficult to satisfy in many cases.

Sisco and Smith (16) showed that among the fragments of *Haemophilus* DNA generated by restriction endonucleases, only certain of them could be taken up by competent recipient cells (as measured by protection from nuclease digestion). Efficient uptake was correlated with the presence in the DNA of a specific 11-base pair

(bp) uptake site. In consideration of these findings, successful construction of recombinant plasmid DNAs bearing transformationally active genetic markers ought to depend on the location of uptake sites and restriction sites, relative to genetic markers, on the *H. influenzae* genome. To retain transforming activity, a restriction fragment must contain, in addition to a genetic marker, at least one uptake site.

The dependence of uptake on the presence of a specific sequence provides a plausible explanation for the earlier finding by Goodgal and Gromkova (7) that different *H. influenzae* genetic markers are inactivated to different extents by a restriction endonuclease from *H. parainfluenzae*. In this paper, we describe the action of various restriction endonucleases on *H. influenzae* transforming DNA with regard to (i) the inactivation of genetic markers and (ii) the lengths of restriction fragments bearing genetic markers.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and genetic transformation. *H. influenzae* wild-type strain Rd was obtained from J. K. Setlow. Strain Rd-8 (1), bearing eight antibiotic resistance markers (*str*, *nov*, *kan*, *nal*,

ery, *stv*, *spc*, and *vio*) was kindly provided by R. F. Kimball, as was *H. influenzae* *thy* *proB2* *str* *nov* *vio*. Cells were grown in Difco brain heart infusion broth (3.7%) supplemented with 10 μ g of hemin per ml (Eastman Organic Chemicals) and 2 μ g of NAD per ml (Sigma Chemical Co.) as described by Setlow et al. (14). Cells were made competent in M-IV medium, and transformation was carried out by the method of Herriott et al. (8). For transformation to drug resistance, cells were diluted in unsupplemented brain heart infusion broth and plated under the surface of solid growth medium (14). After a 90-min expression period at 37°C, plates were overlaid with the appropriate double-strength drug agar to give the following final concentrations of antibiotic: streptomycin, 250 μ g/ml; novobiocin, 2.5 μ g/ml; kanamycin, 7 μ g/ml; and naladixic acid, 3 μ g/ml. For transformation to *Thy*⁺, 2 to 4 volumes of supplemented brain heart infusion broth was added after the DNA uptake period, and cells were grown for 2 to 3 h before being diluted in M₁ medium and plated onto the surface of M₁ agar (9) containing the appropriate supplements (10).

Transforming DNA and restriction endonucleases. DNA was purified from *H. influenzae* strain Rd-8 by the method of Marmur (12) and dissolved in 10 mM Tris-hydrochloride (pH 7.5) 1 mM EDTA, and 50 mM NaCl. DNA concentrations were determined by the diphenylamine assay (6). DNA of bacteriophage lambda was purchased from Bethesda Research Laboratories. Restriction endonuclease *Eco*RI was kindly supplied by A. Dugaiczky. *Bam*HI, *Bst*EII and *Cla*I were purchased from Boehringer-Mannheim. Endonucleases *Msp*I, *Sau*3A, *Pst*I, *Taq*I, and *Pvu*II were obtained from New England Biolabs. Endonucleases *Xho*I, *Ava*II, *Hpa*II, *Hinf*I, *Sal*I, *Hha*I, and *Bgl*II were purchased from Bethesda Research Laboratories.

Measurement of inactivation of transforming activity. *H. influenzae* DNA (1 μ g) from strain Rd-8 was digested for 1 h in 50- μ l mixtures under the conditions recommended by the supplier for each restriction endonuclease. Sufficient enzyme was used to completely digest 2.5 μ g of phage lambda DNA (determined by electrophoresis in 1% agarose, followed by illumination of ethidium bromide-stained DNA with near UV light). At various times, 10- μ l samples (containing 0.2 μ g DNA) were diluted into 100 μ l of 10 mM potassium phosphate (pH 7.5)-5 mM EDTA, and stored at -20°C. To determine the transforming activity of the digested DNA, 50 μ l of diluted digest (0.09 μ g) was added to 2 ml of competent cells (strain Rd for drug markers; *Thy*⁻ cells for *thy* marker), and transformation frequencies were measured as described above.

Determination of lengths of restriction fragments bearing genetic markers. Rd-8 DNA (10 μ g) was digested for 2 h in 50- μ l reaction mixtures. Sufficient enzyme was used to produce maximal inactivation of transforming activity and to completely digest 25 μ g of phage lambda DNA in 1 h. Reactions were terminated by addition of 25 mM EDTA, heated at 65°C for 3 min, put on ice, and mixed with a 10% volume of dye solution (0.1% bromophenol blue, 10 mM EDTA, 10 mM Tris-hydrochloride [pH 7.5], 50% glycerol) and loaded into wells formed near one end of a 14 by 26 cm (300-ml) slab of 0.5% agarose (Sigma grade V) containing 0.5 μ g of ethidium bromide per ml and electropho-

resis buffer (40 mM Tris base, 20 mM sodium acetate, and 1 mM EDTA [pH 7.2]). Electrophoresis was carried out at 50 to 100 V until the dye migrated to near the end of the gel. Gels were photographed under near UV light and then sliced into 24 sections. DNA was extracted from each gel slice by three cycles of freeze-thaw (18). For measurement of transforming activity, 5 μ l of the supernatant from each gel slice was added to 1 ml of competent cells, except for when survival of transforming activity was below 0.1%, in which case 25 μ l of supernatant was added to 1 ml of competent cells. Size markers, electrophoresed adjacent to the digested *H. influenzae* DNA, consisted of phage lambda DNA digested with *Eco*RI (producing fragments of 3.38 to 21.8 kilobases [kb] [19, 20]) or *Pst*I (producing fragments of 1.96 to 11.88 kb [17]). By plotting the electrophoretic mobility of the size markers versus the log of their length, a line was obtained to which the electrophoretic mobility of the peak of transforming activity could be compared to estimate the length of the restriction fragment bearing a genetic marker.

RESULTS

Figure 1 shows the effect on transforming activity that was produced by digestion of *H. influenzae* DNA with six different restriction endonucleases. In cases in which genetic markers were inactivated, transforming activity decreased with time and leveled off at a value of the percentage of survival that is fairly reproducible for each combination of enzyme and marker. In control experiments (data not shown), we showed that the rate of inactivation of transforming activity, but not the final level of survival, was dependent on enzyme concentration.

The extent of marker inactivation depended on both the enzyme used in the digestion and the genetic marker examined. For example, transforming activity of the *nov* marker was inactivated down to about 0.5% survival by *Eco*RI, whereas that of the *kan* marker was nearly unaffected. The reverse was seen with *Hpa*II and *Ava*II, with *nov* relatively unaffected and *kan* inactivated extensively. Similarly, with *Ava*II, *kan* was severely inactivated and *nal* was unaffected, whereas with *Sau*3A, *nal* was inactivated to a level of survival 100-fold lower than that of *kan*.

From the data of Fig. 1 and results found with other restriction endonucleases (data not shown), we found that the greatest extent of inactivation of transforming activity generally occurred with enzymes that are known to produce the smallest fragments (enzymes with four base pair recognition sequences). To know whether exceptions to this generalization exist requires a knowledge of the lengths of restriction fragments carrying genetic markers.

Figure 2 shows the results of experiments in which we estimated the lengths of restriction fragments bearing various genetic markers of *H. influenzae*. For each enzyme, the profile of

FIG. 1. Inactivation of transforming activity of *H. influenzae* genetic markers by restriction endonucleases. Symbols: Δ , *nov*; $\circ$, *str*; $\square$, *kan*; and $\bullet$, *nal*.

transforming activity (obtained with DNA extracted from agarose gel slices) is plotted beneath the corresponding electrophoretic pattern of restriction fragments. In addition to the broad smear of fragments expected for a total genomic length of 2,400 kb (11), each enzyme produced a characteristic banding pattern.

For most restriction endonucleases, there was only one major peak of transforming activity, at a position (relative to the marker fragments) that was reproducible. Enzymes that produced the greatest inactivation of the *nov* marker (e.g., *TaqI* and *PstI*) gave broader distributions of transforming activity throughout the gel. In these cases, the transforming activity seen with the longer fragments is likely to represent transformation by a small population of incompletely digested DNA molecules, which transform at much higher efficiency than the final product of digestion. In the case of the *PstI* digest, multiple peaks of transforming activity were seen with the highly inactivated *nov* marker (Fig. 2A), but single sharp peaks were produced (data not shown) for the larger fragments bearing *str* (3.4 kb), *kan* (3.9 kb), and *nal* (11 kb).

From the experiments of Figure 2 and other work (not shown), we estimated that the lengths of restriction fragments bearing transforming

activity for *nov* ranged from 0.9 kb (*PstI*) to about 25 kb (*BglII*). In some cases (e.g., *TaqI* and *PstI*; Fig. 2A), the peak of transforming activity for *nov* occurred in a region of the gel containing only a small fraction of the digested DNA, which provides considerable enrichment for the *nov* marker. Similarly, with *EcoRI* (Fig. 2B), fragments bearing the *nov*, *str*, and *kan* markers migrated to a position well separated

FIG. 2. Determination of lengths of restriction fragments bearing genetic markers of *H. influenzae*. (A) Electrophoretic mobility of restriction fragments bearing the *nov* marker after digestion with various enzymes. (B) Mobility of fragments bearing various drug resistance markers after digestion with *XhoI* or *EcoRI*. The relative mobility used to calculate the fragment length for each marker is indicated by a vertical line at the top of each peak of transforming activity. Beneath the photographs of ethidium bromide-stained DNA are shown the positions of size markers, which consisted of bacteriophage lambda DNA digested with *EcoRI* (21.80, 7.55, 5.93, 5.54, 4.80 and 3.38 kb, from left to right [19, 20]). For genetic marker-bearing fragments shorter than 5 kb, *PstI* fragments of lambda DNA served as smaller size markers.

TABLE 1. Action of restriction endonucleases on transforming DNA of *H. influenzae*^a

Genetic marker	Restriction endonuclease	% Survival of transforming activity	Length (kb) of marker-bearing fragment
<i>nov</i>	None	100	50
	<i>Hpa</i> II	100	13
	<i>Bgl</i> III	84	25
	<i>Ava</i> II	80	17
	<i>Pvu</i> II	30	9.8
	<i>Xho</i> I	20	7.5
	<i>Cla</i> I	10	7.5
	<i>Eco</i> RI	0.5	1.7
	<i>Sau</i> 3A	0.07	1.3
	<i>Pst</i> I	0.02	0.9
	<i>Taq</i> I	0.009	4.0
	<i>Hin</i> fI	0.005	2.8
<i>str</i>	<i>Xho</i> I	20	13
	<i>Cla</i> I	8	5.0
	<i>Ava</i> II	3	3.1
	<i>Pst</i> I	3	3.4
	<i>Hpa</i> II	0.06	0.9
	<i>Eco</i> RI	0.04	2.2
<i>kan</i>	<i>Cla</i> I	100	27
	<i>Xho</i> I	90	13
	<i>Eco</i> RI	50	2.1
	<i>Pst</i> I	4	3.9
	<i>Hpa</i> II	0.7	2.3
	<i>Ava</i> II	0.3	— ^b
<i>nal</i>	<i>Xho</i> I	100	28
	<i>Hpa</i> II	100	20
	<i>Ava</i> II	100	26
	<i>Eco</i> RI	70	11
	<i>Cla</i> I	50	12
	<i>Pst</i> I	36	11
<i>thy</i>	<i>Xho</i> I	50	34
	<i>Ava</i> II	35	25
	<i>Eco</i> RI	5	13

^a Data were compiled from the experiments of Fig. 1 and 2 plus unpublished experiments. When applicable (*nov* marker with *Sau*3A, *Pst*I, *Taq*I, and *Hin*fI, *str* marker with *Eco*RI and *Hpa*II), values for survival of transforming activity were adjusted downward to correct for transforming activity of products of incomplete digestion.

^b —, Peak of transforming activity was not detected over the background of spontaneous mutagenesis.

from the bulk of the digested DNA. Our estimate of the length of the *Pvu*II-*nov* fragment (9.8 kb) is close to the size of the inserted *Pvu*II fragment of *H. influenzae* DNA in a *nov*-bearing recombinant plasmid (9.4 kb) as estimated by electron microscopy (15). Also, the value that we determined for the length of the *Pst*I-*nov* fragment (0.9 kb, Fig. 2A) has been verified by subcloning a *nov*-bearing *Pst*I fragment from that same recombinant plasmid (J. K. Setlow, personal communication).

For *Xho*I, the *str* and *kan* markers appear to

be present on the same 16-kb *Xho*I fragment, since (i) they appeared at the same position in the agarose gel (Fig. 2B) and (ii) the *Str*^r*Kan*^r double transformants also appeared at 16 kb (data not shown) at a frequency that was very close to that for *str* alone and orders of magnitude greater than expected for independent recombinational events. In the case of *Eco*RI, although *kan* and *str* were both about 2.2 kb (Fig. 2B), these markers are probably present on separate *Eco*RI restriction fragments, since (i) transformation to *Kan*^r with DNA from gel slice 20 was about 200-fold more frequent than that to *Str*^r and (ii) the frequency of transformation to *Kan*^r*Str*^r was 50-fold lower than that to *Str*^r alone and very close to that expected for two independent recombinational events.

Table 1 is a compilation of all our data on the action of restriction endonucleases on *H. influenzae* transforming DNA. The data were taken from Fig. 1 and 2 and from unpublished experiments. From Table 1, it is evident that loss of transforming activity is associated with a decrease in the length of the restriction fragment bearing a given genetic marker. This is graphically demonstrated in Fig. 3. Here we have plotted, for each genetic marker, the fraction of transforming activity remaining after digestion with each restriction endonuclease versus the length of the marker-bearing restriction fragment produced by the corresponding enzyme. The line in Fig. 3 is meant to represent the results obtained for *nov*, the marker which we examined most thoroughly. The transforming activity dropped off dramatically as the length of the restriction fragment fell below 10 to 15 kb. A decrease in length from 10 to 1 kb was associated with more than a 1,000-fold decrease in transforming activity. Most of the data for other genetic markers fell quite close to the line representing the *nov* marker. Also plotted are points calculated from published data of Randolph and Setlow (13) on the inactivation of transforming activity (*str* marker of *H. influenzae*) by sonic radiation, which produced random double-strand breaks in the DNA. These data, for which fragment lengths were calculated from weight-average molecular weights, fell remarkably close to our data obtained with restriction endonucleases. The available data for the *thy* marker suggest a higher molecular weight threshold for inactivation of *thy* transforming activity than with other markers. The positioning of a few points well above or below the line indicates that length alone is not the only factor determining the transforming activity of a restriction fragment bearing a genetic marker.

DISCUSSION

Inactivation of transforming activity of *H. influenzae* DNA by restriction endonucleases

FIG. 3. Relationship between fragment length and survival of transforming activity. Data from Table 1 were used to plot the surviving fraction of transforming activity (S/S_0) versus the length (L) of the restriction fragment bearing a given genetic marker. Symbols: Δ , *nov*; $\circ$, *str*; $\square$, *kan*; $\bullet$, *nal*; $\blacktriangle$, *thy*; +, undigested DNA ($S/S_0 = 1.00$); $\diamond$, data of Randolph and Setlow (13) for S/S_0 (*str*) versus weight-average molecular weight after random shearing by sonic radiation.

must at least partially be caused by effects on DNA uptake into competent cells, since efficient binding of restriction fragments to competent cells of *H. influenzae* (in a form resistant to enzymatic hydrolysis and high salt elution) is associated with the presence of specific uptake sites (3). DNA from unrelated species contains very few of the uptake sites and is taken up poorly by competent *H. influenzae* cells (2, 4). Whereas the *E. coli* plasmid pBR322 is not taken up by competent *H. influenzae* cells, ligation of specific *Haemophilus* sequences into this DNA leads to efficient uptake (2, 16). Similarly, insertion of *Haemophilus* chromosomal DNA into the plasmid RSF0885 results in efficient uptake associated with high frequency of transformation (15). Insertion of chemically synthesized 11-bp uptake sites (AAGTGGGTC) into plasmid pBR322 also promotes efficient uptake into competent cells; the efficiency at which an uptake site promotes entry of DNA into competent cells is strongly influenced by the nucleotide composition flanking the 11-bp sequence (3a). Variant 11-bp sequences may also exist which promote uptake at a lower efficiency (5). Efficient uptake may even be conferred by specific sequences different from the 11-bp segment referred to above (S. Goodgal, personal communication).

The above results lead one to expect that a large factor determining the transforming activity of a genetic marker of *H. influenzae* following cleavage by a restriction endonuclease is the positioning of uptake sites in the region of the marker, relative to the cleavage sites. The length (10 kb) below which we observed a sharp drop in transforming activity is not too different from the predicted 4-kb average spacing for high efficiency uptake sites (16). If a restriction endonuclease cleaves the DNA between a marker and the flanking uptake sites, its transforming activity will be destroyed because of its failure to be taken up by recipient cells. If the restriction endonuclease unlinks the marker from nearby high-efficiency uptake sites, leaving a fragment containing the marker plus lower efficiency uptake sites, the transforming activity should be partially inactivated. Thus, the 10,000-fold range in transforming activity observed for marker-bearing fragments of length 1 to 4 kb (Fig. 3) may reflect the relative positioning along the genome of uptake sites, restriction sites, and genetic markers. Other factors that might affect transformation efficiency of a marker-bearing fragment include (i) the distance between a marker and flanking uptake sites, (ii) the orientation of the asymmetric 11-bp uptake sequence with respect to the position of the genetic marker, and (iii) the distance between the marker and the ends of the DNA molecule. Whether any of these factors are important, at the level of DNA uptake or at later stages in transformation, will be subject to biochemical examination after recombinant DNAs are constructed which carry the appropriate genetic markers.

The data presented in this paper should aid in the construction of recombinant DNAs containing *H. influenzae* genetic markers. The availability of these DNAs will allow simultaneous genetic and biochemical analyses not only of genetic transformation, but also of other processes, including DNA repair and mutagenesis.

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