Comparison of Transformation Mechanisms of *Haemophilus parainfluenzae* and *Haemophilus influenzae*

FRANCIS BARANY* AND MARC E. KAHN

Department of Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received 21 August 1984/Accepted 6 October 1984

Transformation pathways in two closely related bacterial species, *Haemophilus parainfluenzae* and *Haemophilus influenzae*, were studied. Both organisms rapidly take up transforming DNA within minutes into specialized membranous structures on the cell surface (transformasomes). DNA within transformasomes is in a protected state, inaccessible to external DNase or internal restriction and modification enzymes. However, the subsequent processing of donor DNA differs in these two organisms. In *H. influenzae*, linear DNA immediately undergoes degradation from one end at a constant rate, leaving a lower-molecular-weight intermediate in the transformasome. The end undergoing degradation is searching for homologous regions of the chromosome. Once pairing is initiated, the remaining lower-molecular-weight DNA exits from the transformasome, and a single strand undergoes efficient integration. In contrast, in *H. parainfluenzae* little degradation of donor DNA is observed, with the majority remaining intact within the transformasomes after 1 h. Thus, whereas only 10% of donor DNA molecules leave the protected state after 1 h, portions of each molecule appear to become quantitatively integrated.

Haemophilus influenzae and *Haemophilus parainfluenzae* are genetically closely related gram-negative bacteria that differ in pathogenicity, hemin requirements, restriction enzymes, and transformation pathways (7, 8, 12, 27). Both *Haemophilus* species have evolved mechanisms for taking up and integrating transforming DNA (for reviews, see references 9, 12, 19a, 21, 29, and 34). The ability of bacteria to take up DNA, known as the competent state, is characterized by several changes in cell physiology and membrane structure (18, 19, 39, 43). Recently, Kahn et al. have shown that both *Haemophilus* species synthesize about 10 to 12 specialized membranous extensions (transformasomes) on their surface during the onset of competence (18, 19) (Fig. 1A). Upon the addition of homologous donor DNA, transformasomes are internalized into the cytoplasm in *H. parainfluenzae*, but remain on the surface of *H. influenzae* (Fig. 1B).

H. influenzae and *H. parainfluenzae* are able to share genetic information through transformation. Each species possesses a powerful restriction and modification system that attacks the DNA of unmodified infecting bacteriophages, reducing infection by 10^3 - to 10^9 -fold (38). These systems are distinct and confer different methylation patterns on the host DNA. However, naked phage or bacterial DNAs (which are not properly modified) are not restricted when transformed between *H. influenzae* and *H. parainfluenzae* (14, 38). Since donor DNA remains double stranded upon uptake in *Haemophilus* species (37, 40), it appears paradoxical that unmodified donor DNA can escape restriction.

We have recently shown that *H. influenzae* takes up donor DNA into transformasomes, where it is in a protected state, untouched by cellular restriction and modification enzymes (2, 12). The fate of donor DNA is dependent on its topological conformation; circular DNA remains intact within transformasomes, whereas linear DNA is degraded to a lower-molecular-weight intermediate during exit. Linear donor

DNA apparently undergoes directional exit from the transformasome through a pore at the site of membrane attachment, a process which we term translocation (2). The 5' strand is preferentially degraded, presumably leaving a 3' single-stranded tail that is resistant to restriction, and can undergo homologous integration into the chromosome (2).

In this work we demonstrate that *H. parainfluenzae*, like *H. influenzae*, protects unmodified donor DNA from restriction enzymes by uptake of DNA into transformasomes. In contrast to *H. influenzae*, donor DNA within transformasomes in *H. parainfluenzae* either remains intact or is degraded at a far slower rate. Nevertheless those molecules that undergo translocation are efficiently integrated into the chromosome.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *H. influenzae* Rd strain KW22 (42) was grown in 2.5% heart infusion broth (Difco Laboratories) supplemented with hemin (Eastman Chemical Products, Inc.) at 10 µg/ml and NAD (Sigma Chemical Co.) at 2 µg/ml. *H. parainfluenzae* strain 14 (18) was grown in 3.9% brain heart infusion broth (Difco) and supplemented as above. Competent *H. parainfluenzae* cells were prepared by the procedure of Gromkova and Goodgal (15), whereas *H. influenzae* cells were made competent by the MIV procedure of Herriott et al. (16). *Escherichia coli* strains HB101 *hsdS recA* and MM294 *endA hsdR* containing plasmids were grown in L broth (25). Plasmids were prepared by the alkaline procedure of Birnboim and Doly (4) and further purified by one or two CsCl-ethidium bromide centrifugation (30). Plasmid pCML6 contains a 10-kilobase (kb) insert of *H. influenzae* Rd DNA in the *Bam*HI site of a pBR322 derivative (6, 17). Plasmid pKS17 contains an 8-kb *Bam*HI fragment of *H. parainfluenzae* DNA in the *Bam*HI site of pBR322 (32). Plasmid pPUP3 contains an 11-base-pair "uptake sequence" within the *Pst*I site of pBR322 (6).

Preparation of 32 P-labeled DNA. Circular plasmid DNA (5 µg of pPUP3, pCML6, or pKS17) was "pre-nicked" in a

* Corresponding author.

FIG. 1. Structural analysis of *H. parainfluenzae* transformasomes by electron microscopy. Thin sections of competent *H. parainfluenzae* cells demonstrate transformasomes on the cell surface (arrows). After the addition of transforming DNA (B), surface transformasomes are no longer observed, whereas vesicles accumulate in the periplasm, sometimes associated with cytoplasmic membrane (arrow). Vesicles are not observed in the periplasm of *H. influenzae* cells after the addition of transforming DNA (19). Bar, 100 nm.

volume of 50 μ l, containing 50 mM Tris-hydrochloride (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, and 7 mM mercaptoethanol (TEMM buffer) with 3×10^{-6} mg of pancreatic DNase (Bethesda Research Laboratories) per ml by incubating at 23°C for 5 min. DNase digestion was terminated by heat treatment at 65°C for 5 min. Under these conditions about 80% of the plasmid was converted to open circles, about 10% was converted to the linear form, and 10% remained as covalently closed circles, as judged by electrophoretic mobility in agarose gels. Nick translation of 2 μ g of prenicked DNA was carried out in a volume of 40 μ l of TEMM buffer containing 300 μ M each dATP, dGTP, and dTTP and 20 μ Ci of [α - 32 P]dCTP by incubating with 1 U of *Micrococcus luteus* polymerase (Miles Laboratories, Inc.) at 15°C for 5 min. Half of the reaction was removed and terminated with 100 mM EDTA, yielding labeled open circular DNA. Unincorporated label was removed by chromatography on a Sephadex G25 column (Pharmacia; lot T24591) preequilibrated with no. 21 medium (16). The remaining DNA was sealed by the addition of 600 μ M ATP, 300 μ M dCTP, and 1 Weiss unit of T4 ligase (New England Biolabs) for 5 min at 15°C. The reaction was terminated with 100 mM EDTA, and DNA was purified on G25 as described above. The DNA specific activity averaged 2×10^7 cpm/ μ g. More than half of the strands were intact as judged by denaturation and electrophoresis (see Fig. 5, lanes A through D). Nick translation occasionally leaves a 1- or 2-base 5' displaced strand overhang that cannot be ligated (24). With a small circular DNA, such as pPUP3, some resealed circular DNA topoisomers migrate faster than open circular DNA and are seen as a multitude of bands (see Fig. 3, lane D). However, with larger DNAs (pKS17 and pCML6), resealed circular DNA migrates as relaxed DNA under the electropho-

retic conditions used here (see Fig. 3, lane A, and Fig. 5, lanes C and D).

Sealed circular plasmids were linearized by the addition of 10 mM $MgCl_2$ and 50 mM NaCl to the DNA in no. 21 medium and incubation with *Pvu*I or *Nru*I for 10 min at 37°C just before the DNA uptake assay. *Cla*I linear nick-translated pCML6 was prepared as described (17).

Restriction enzyme digestions, gel electrophoresis, and blot hybridizations. Restriction endonucleases were from New England Biolabs or Boehringer Mannheim and were used as recommended by the manufacturers. Genomic and plasmid DNA restriction fragments were separated by electrophoresis on 0.8% agarose gels in 0.2 M glycine-NaOH (pH 8.5) at 6 V/cm for 2 h. Gels were dried on DE81 paper and autoradiographed on Kodak XAR-5 film (usually overnight, 12 to 16 h). Hybridization was performed as described by Southern (35) and modified by Wahl et al. (41), with fragments transferred to nitrocellulose as described by Smith and Summers (33).

Donor DNA uptake, reisolation of total DNA, and quantitation of results. Nick-translated DNA was added to 2 ml of competent *H. influenzae* cells in MIV medium or 1 ml of competent *H. parainfluenzae* cells in brain heart infusion broth, and the mixture was allowed to incubate for various times at 37°C. Less than one DNA molecule was added per cell (50 ng is 3.0×10^9 molecules and 6×10^5 cpm to approximately 4×10^9 to 6×10^9 cells). DNA uptake was 21 to 33% in *H. parainfluenzae* and 50 to 80% in *H. influenzae*. Transformation was terminated by chilling cells to 0°C, and excess DNA was removed by digestion with 1 mg of pancreatic DNase per ml for 20 min at 0°C. Cells were lysed in 10 mM Tris-hydrochloride (pH 8.0) containing 10 mM EDTA, 0.1 M NaCl, and 1% sodium dodecyl sulfate (SDS);

FIG. 2. Restriction maps of plasmids pKS17 and pCML6 and surrounding regions in *H. parainfluenzae* and *H. influenzae* chromosomes, respectively. Plasmid pKS17 contains a 8-kb *Bam*HI fragment of *H. parainfluenzae* DNA (32), whereas plasmid pCML6 contains a 10-kb *Sau*IIIA fragment of *H. influenzae* DNA (17) cloned into the *Bam*HI site of pBR322 and a derivative, respectively. The pBR322 region is shaded. In the *H. parainfluenzae* chromosome, the 10.0-kb *Eco*RV and 6.5-kb *Eco*RI junction fragments include the left side of pKS17. In the *H. influenzae* chromosome, the 12.5-kb *Bst*EII junction fragment (J1) includes the left side of pCML6, whereas the 7.0-kb *Bst*EII junction fragment (J2) includes the right side of pCML6 as drawn.

Pierce Chemical Co.) for 5 min at 63°C and incubated for an additional hour at 37°C in the presence of 1 mg of proteinase K (Boehringer Mannheim) per ml. DNA was isolated by two extractions with phenol, two extractions with *n*-butanol, and ethanol precipitation. After rinsing, the precipitate was dissolved in 10 mM Tris-hydrochloride (pH 8.0)–1 mM EDTA. By this technique, 89% of the radioactivity taken up by the cells was recovered. For restriction digests of reisolated DNAs, we used 10 to 20 U of enzyme per µg of DNA to insure complete digests, as determined by ethidium bromide staining and Southern analysis. Approximately 5,000 cpm was applied to each lane of a 1% agarose gel. The developed autoradiograph was used as a template for excising bands of interest, which were quantitated by liquid scintillation counting for 10 min.

RESULTS

Competence development in *H. parainfluenzae* and *H. influenzae*. When comparing the fate of radioactive DNA during transformation in *H. parainfluenzae* to uptake in *H. influenzae*, it would have been desirable to make both species of cells competent by the same technique. However, this was not possible since the MIV protocol (16) resulted in a 2-log decrease in viability of *H. parainfluenzae*, and the overnight incubation protocol of Gromkova and Goodgal (15) also caused a severe loss of viability in *H. influenzae*. High levels of competence in *H. parainfluenzae* could not be consistently achieved by the method of Goodgal designed for *H. influenzae* (11), in contrast to previous results (3). Therefore those conditions which gave the highest levels of competence, namely, MIV for *H. influenzae* and static

overnight incubation for *H. parainfluenzae*, were used. Observed transformation frequencies for a chromosomal marker were 0.8% for *H. parainfluenzae* and 2% for *H. influenzae*.

Structures of plasmids used to study DNA uptake and integration. To follow homologous integration of donor DNA, we used cloned *H. parainfluenzae* and *H. influenzae* DNA. Restriction maps of the two pBR322 derivatives, pKS17, containing 8 kb of *H. parainfluenzae* DNA, and pCML6, containing 10 kb of *H. influenzae* DNA, are shown in Fig. 2. Restriction maps of the surrounding chromosomal DNA in the host organism are shown below in plasmids in Fig. 2. The chromosomal maps were determined by Southern analysis with single or double digests of chromosomal DNA (data not shown). A junction fragment is a restriction fragment on the plasmid border, such that one end of the fragment is within the cloned piece, whereas the other end is found on the chromosome. If the plasmid contains only one site for that enzyme within the cloned DNA, i.e., *Eco*RV in pKS17 or *Bst*EII in pCML6, then both fragments become junction fragments upon homologous integration of donor DNA.

Initial fate of plasmid DNA after uptake in *H. parainfluenzae*. We compared the fate of radioactively labeled, heterologous (pPUP3) as well as homologous (pKS17) plasmid DNA conformers after 1 h of uptake in *H. parainfluenzae*. These DNA molecules were grown in *E. coli* and hence were susceptible to *in vitro* digestion with the *H. parainfluenzae* restriction endonuclease *Hpa*II. Regardless of DNA conformation or content of homologous sequence, approximately 90% of the reisolated label represented intact donor DNA

FIG. 3. Autoradiogram showing the fate of homologous and heterologous DNA in *H. parainfluenzae*. Nick-translated pKS17 (homologous) and pPUP3 (heterologous) (50 ng = 6×10^5 cpm) were added to 1 ml of competent *H. parainfluenzae* cells. After 1 h, from 21 to 33% of donor DNA was taken up. DNA was reisolated and electrophoresed as described in the text. Lanes A through E contained input DNA: (A) circular pKS17, (B) *NruI* linear pKS17 (heterologous ends), (C) *PvuI*-digested pKS17 (two sites, one of which is within *H. parainfluenzae* DNA, leaving homologous ends), (D) covalently closed circular and open circular pPUP3, (E) *PvuI* linear pPUP3. Lanes A' through E' contain reisolated DNA after 1 h of uptake of input DNA in lanes A through E, respectively.

(Fig. 3). Hence donor DNA was not restricted by endogenous *HpaII*. This donor DNA was susceptible to *in vitro* digestion with *HpaII* (Fig. 4, lanes E, G, and H), indicating that it was not methylated. Thus this reisolated DNA is in the protected state, untouched by external DNase or internal restriction and modification enzymes.

Both *H. parainfluenzae* and *H. influenzae* protected donor DNA from restriction and modification, but only in *H. parainfluenzae* did linear DNA remain largely within the

protected state. In *H. influenzae*, linear DNA rapidly exited from the protected state and underwent degradation (2, 17). In both organisms, circular DNA could not efficiently exit from the protected state (Fig. 3, lanes A, D, F, and I; Fig. 5, lanes A, C, E, and F).

Homologous donor DNA that exists from *H. parainfluenzae* transformasomes integrates efficiently into the chromosome. We compared integration of donor DNA containing homologous versus heterologous DNA ends after 1 h of uptake in *H. parainfluenzae*. The results in Fig. 3 demonstrate that in all cases, approximately 5 to 10% of the reisolated label was in the chromosome. The heterologous DNA that exited the transformasome was degraded and randomly reincorporated (data not shown), whereas homologous pKS17 DNA recombined into the chromosome.

Homologous integration was demonstrated by digesting reisolated DNA (Fig. 4, lanes G, K, and P) with *EcoRI* or *EcoRV* (Fig. 4, lanes I, L, N, Q, and S). A new, faintly labeled fragment (Fig. 4, triangle) of higher molecular weight appeared in these digests; this fragment was not observed when starting DNA was digested by these enzymes (Fig. 4, lanes A through D). These higher-molecular-weight fragments were junction fragments (size confirmed by Southern analysis; Fig. 2), and represented homologous integration. Since donor DNA was sensitive to *in vitro HpaII* (Fig. 4, lanes E, F, and H), digestion of reisolated DNA with both *HpaII* and *EcoRI* (or *EcoRV*) could unambiguously separate unintegrated donor DNA from the junction fragments (Fig. 4, lanes J, M, O, R, and T). Once integrated, the radioactive donor DNA localized in junction fragments was methylated (or remained hemimethylated, assuming single-stranded integration) and hence resistant to *in vitro HpaII*. Donor pKS17 DNA, which was methylated with *HpaII* methylase *in vitro* (before transformation), demonstrated similar inte-

FIG. 4. Autoradiogram demonstrating homologous integration in *H. parainfluenzae*. Covalently closed circular (CCC), *NruI* linear, and *PvuI* pKS17 was added to *H. parainfluenzae* and reisolated after 1 h of uptake (Fig. 3). Lanes A through F contain control digests of input DNA: (A) *EcoRI-NruI* pKS17, (B) *EcoRI-PvuI* pKS17, (C) *EcoRV-NruI* pKS17, (D) *EcoRV-PvuI* pKS17, (E) *HpaII-EcoRI-PvuI* pKS17, (F) *HpaII-EcoRV-PvuI* pKS17. Lanes G through T contain reisolated DNA after 1 h of uptake by the DNAs indicated above the lanes: (G) CCC pKS17; uptake, (H) digested with *HpaII* (donor DNA is digested, but integrated DNA is methylated or at least hemi-methylated) and hence resistant to *HpaII*, (I) digested with *EcoRI* (J) digested with *HpaII-EcoRI*; (K) *NruI* linear pKS17 uptake (L) digested with *EcoRI*, (M) digested with *HpaII-EcoRI*, (N) digested with *EcoRV*, (O) digested with *HpaII-EcoRV*; (P) *PvuI* pKS17 uptake (Q) digested with *EcoRI*, (R) digested with *HpaII-EcoRI*, (S) digested with *EcoRV*, (T) digested with *HpaII-EcoRV*. Open triangles show the positions of *EcoRV* (10.0-kb) and *EcoRI* (6.5-kb) junction fragments. The amount of label in a junction fragment was approximately 1 to 2% of the total label in that lane (Table 1).

TABLE 1. Integration of homologous DNA in *H. parainfluenzae*^a

pKS17 donor DNA conformation	Reisolated DNA; % of total label (cpm) found in:				% of chromosomal label representing homologous integration			
	Transformosome	Chromosome	<i>EcoRI</i> junction fragment ^d	<i>EcoRV</i> junction fragment ^d	Theoretical ^b		Experimental ^c (% of theoretical)	
					<i>EcoRI</i> junction fragment	<i>EcoRV</i> junction fragment	<i>EcoRI</i> junction fragment	<i>EcoRV</i> junction fragment
CCC ^e	90 (2,517)	10 (283)	2.2 (62)		20		22 (110)	
<i>NruI</i> linear	92 (3,185)	8 (290)	1.4 (47)	2.1 (75)	20	34	16 (80)	26 (76)
<i>PvuI</i> (two fragments)	95 (3,796)	5 (192)	1.2 (50)	1.2 (48)	20	22	25 (125)	24 (109)

^a The experiment is described in the legends to Fig. 3 and 4. Relevant bands were excised (Fig. 3, lanes A', B', C', D', and E'; Fig. 4, lanes G, H, J, K, M, O, P, R, and T) and counted.

^b The theoretical percentage of label representing homologous integration was calculated by assuming that nick translation of starting pKS17 was uniform; i.e., the theoretical value for *EcoRI* junction was (length of fragment/length of starting DNA) $\times$ 0.5 (only one strand integrates) = (5/12.4) $\times$ 0.5 = 20%.

^c Experimental values were calculated by dividing the amount of label in the junction fragment by the total label found in the chromosome. In all cases experimental values approached theoretical ones.

^d *EcoRI* junction fragment is 6.5 kb, and the *EcoRV* junction fragment is 10 kb (Fig. 2). Numbers within parentheses have been corrected for random chromosomal incorporation (22 cpm).

^e CCC, Covalently closed circle.

gration of label into junction fragments, as did unmethylated pKS17 DNA (data not shown). These results suggest that processing of DNA during transformation and integration, in both *H. parainfluenzae* and *H. influenzae* (17), is unaffected by the lack of protective donor DNA methylation.

The extent of homologous integration was determined by excising from the gel those bands corresponding to junction fragments and determining their radioactivity in a scintillation counter (Table 1). Only a minority of the total label was redistributed into the chromosome (5 to 10%); even less appeared in the junction fragments (1 to 2%) (Table 1). Therefore approximately 20% of the chromosomal label

represented a junction fragment. Twenty percent approaches the theoretical percentage of the donor molecule that could integrate into the junction fragment (subtracting the heterologous DNA and assuming single-stranded integration). Therefore, independent of donor DNA conformation, whether predominantly covalently closed circular (pKS17), linear with heterologous ends (*NruI* pKS17), or linear with homologous ends (*PvuI* pKS17), the homologous regions of those molecules that underwent translocation appeared to be efficiently integrated into the chromosome. The ability of circular pKS17 to exit from transformosomes (translocate) and become integrated may be due to nicking (or linearization) of circles and exiting at a nick (or end), or to preferential exiting of minor amount of linear molecules present in the input circular DNA (Fig. 3, lanes A, A', D, and D').

Integration of homologous DNA in *H. influenzae* is efficient, but is accompanied by considerable degradation at the integrating strand. In *H. influenzae*, radioactively labeled homologous pCML6 circular DNA remained within transformosomes (just as heterologous circular DNA) after 1 h of uptake (Fig. 5, lane F). However, linear donor DNA (*ClaI*-cut pCML6) rapidly exited from transformosomes, and after 1 h all of the label was reincorporated into the chromosome (Fig. 5, lane E) (2, 17). Analysis of the chromosomal label by *EcoRI* or *BstEII* digestion revealed that the majority of label was randomly distributed (Fig. 5, lanes G through M). However, a minority of label was found at the proper junction fragments (Fig. 5, dots), indicating homologous integration.

The extent of proper integration was quantitated by excising and counting the unique *BstEII* junction fragments and correcting for background random incorporation (Table 2; Fig. 5, lanes K and L). Junction fragment J1 (12.5 kb)

FIG. 5. Autoradiogram demonstrating degradation and homologous integration of donor DNA in *H. influenzae*. Nick-translated pCML6 (50 ng = 10^6 cpm) was added to 4.6×10^9 competent KW22 cells (less than one DNA molecule per cell), and DNA was reisolated after 60 min of uptake. Lanes: (A) input *ClaI* linear pCML6; (B) heat-denatured *ClaI* linear pCML6, demonstrating that the majority of nicks were sealed to full length linear DNA; (C) input circular pCML6; (D) heat-denatured circular pCML6. About half of the circular DNA was covalently closed. (E through F) Reisolated DNA after uptake of (E) *ClaI* linear pCML6 and (F) circular pCML6. Lanes G through J contained *EcoRI* digests of (G) *ClaI* linear pCML6, (H) reisolated DNA after uptake of *ClaI* linear DNA, (I) circular pCML6, and (J) reisolated DNA after uptake of circular pCML6. Solid diamond dots indicate the positions of chromosomal fragments that have homology to pCML6. Only the 4.6-kb fragment in lane H shows appreciable specific incorporation (homologous

recombination) above background. Lanes K through M contain 20,000 cpm per lane: *BstEII* digest of (K) reisolated DNA after uptake of *ClaI* linear pCML6, (L) reisolated DNA after uptake of random 14-kb DNA, (M) *ClaI* pCML6. Solid diamond dots indicate the positions of *BstEII* junction fragments J1 (12.5 kb) and J2 (7.0 kb). The lane L autoradiogram is identical to an ethidium bromide stain of chromosomal DNA digested with *BstEII*. The amount of label in the correct junction fragment (lane K) minus randomly incorporated label for that size class (lane L) was used to calculate the amount of homologous recombination (Table 2).

TABLE 2. Integration of homologous DNA in *H. influenzae*^a

pCML6 Donor DNA	Reisolated DNA; % of total label (cpm) found in:				% of chromosomal label representing homologous integration			
	Transformasome	Chromosome	<i>Bst</i> EII junction		Theoretical ^b <i>Bst</i> EII junction		Experimental ^c (% of theoretical <i>Bst</i> EII junction)	
			Fragment J1 ^d	Fragment J2 ^d	Fragment J1	Fragment J2	Fragment J1	Fragment J2
<i>Cla</i> I linear	15 (1,293)	85 (7,485)	5.2 (460)	8.8 (773)	15.3	19.4	6.1 (40)	10.3 (53)

^a The experiment is described in Fig. 5. Relevant bands were excised (Fig. 5, lanes K and L) and counted.

^b Theoretical percentage of label representing homologous integration was calculated by assuming that nick translation of starting pCML6 was uniform; i.e., the theoretical value for J2 junction was (length of fragment/length of starting DNA) $\times$ 0.5 (only one strand integrates) = $5.6/14.4 \times 0.5 = 19.4\%$.

^c Experimental values were calculated by dividing the amount of label in the junction fragment by the total label found in the chromosome.

^d The *Bst*EII junction fragment J1 is 12.5 kb; J2 is 7.0 kb (Fig. 2). Numbers within parentheses have been corrected for random chromosomal incorporation (398 cpm in J1 region and 320 cpm in J2 region).

contained 5.2%, and J2 (7.0 kb) contained 8.8%, representing 14% of the total label reincorporated into the chromosome. Since only a single strand is thought to integrate (28), and 70% of pCML6 is homologous DNA, only 35% of the starting donor label could represent homologous integration into the junction fragments. Since donor DNA is also degraded from an end, even less label would integrate into the proper junction fragment. The difference in label incorporated into the two junction fragments J1 and J2 can be accounted for by degradation of donor DNA from an end. Calculations of the efficiency of integration of the J2 fragment suggest that either about one out of every two molecules underwent homologous recombination or all participated and only a portion was integrated: (experimental incorporation of J2)/(maximum theoretical incorporation of J2) = $10.3/19.4 = 53\%$. Thus, somewhere from 53 to 100% of the molecules that were translocated underwent homologous integration. This is similar to the result in *H. parainfluenzae*, where a high percentage of molecules that exited from the transformasome underwent homologous recombination.

DISCUSSION

We have followed the fate of radioactivity labeled donor DNA during transformation in *H. parainfluenzae* and compared it with our previous results in *H. influenzae*. Both organisms take up donor DNA into transformasomes (18, 19), where it is protected from intracellular restriction and modification enzymes. In this protected state, the donor DNA remains double stranded (2, 17, 31, 37, 40). The DNA is physically within the cells, as evidenced by resistance to exogenously added DNase or high-salt washing. Subsequently, donor DNA translocates out of the transformasomes, and integration is initiated. In this work, we have attempted to quantitate the integration of donor DNA into the chromosome. In *H. parainfluenzae*, after 60 min, only a small percentage of donor molecules exit from the transformasomes. Consequently the amount of radioactivity within junction fragments is low, but has been reproduced several times. In *H. influenzae*, degradation of donor DNA is extensive and responsible for a high background of randomly incorporated label; however, junction fragments reproducibly appear in kinetic experiments and are intensified by using middle-labeled donor DNA (2). Nonetheless, within the limits of this biochemical technique (50% error), we conclude that in both organisms, portions of each molecule that undergoes translocation become integrated.

One of the striking differences between *H. parainfluenzae* and *H. influenzae* is the morphological fate of transfor-

masomes. After the addition of transforming DNA, the transformasomes move as intact particles into the periplasm and cytoplasm of *H. parainfluenzae*, whereas in *H. influenzae*, the transformasomes remain on the surface (19). The biochemical results reported here are consistent with differences in the transformation frequencies observed and may be related to the fate of the DNA-transformasome complex. In *H. parainfluenzae*, the majority of donor DNA remains intact in the transformasomes 60 min after uptake, and consequently extensive degradation of donor DNA is not observed. Electron microscopic analysis of DNA reisolated from *H. parainfluenzae* transformasomes revealed some full-length molecules with a short region of single-stranded DNA at one end (M. Kahn, unpublished data). In contrast, in *H. influenzae* all linear donor DNA molecules progressively move out of the transformasomes and undergo extensive degradation from an end. Analysis of DNA reisolated from *H. influenzae* transformasomes revealed that many molecules were smaller than full-length donor DNA (2, 17). A free end is necessary for degradation, since covalently closed circular donor molecules remain intact (after 60 min) in both species.

Before integration, donor DNA is in a double-stranded, somewhat degraded form, residing within the transformasomes of both species. Portions of the donor DNA, in particular a single-stranded end, may be able to initiate homologous recombination. During the search for homology, the single-stranded end appears to undergo less degradation in *H. parainfluenzae* than in *H. influenzae*. We speculate that in *H. parainfluenzae* the internalized transformasome-DNA complex is actively "searching" for chromosomal homology. In *H. influenzae*, however, the transformasomes remain fixed on the outer membrane. Hence, integration of a donor DNA molecule may be dependent on the proximity of homologous chromosomal sequences to that DNA-containing transformasome. Translocating donor DNA undergoes continuous degradation until it finds homology. If homologous pairing is initiated, the surviving portion of the donor DNA molecule can undergo efficient integration.

It has been shown genetically that both gram-negative and gram-positive bacteria severely restrict infecting DNA, whereas transforming DNA is not restricted (1, 5, 14, 26, 36, 37). In gram-positive bacteria, a membrane-bound endonuclease degrades one strand of donor DNA, whereas the other strand is internalized (20, 22, 23); thus transforming DNA avoids restriction by virtue of its single strandedness. In contrast, the gram-negative bacteria *H. influenzae* and *H. parainfluenzae* initially protect double-stranded donor DNA in transformasomes. In *H. influenzae* and the gram-positive,

transformable bacteria, only a single strand of donor DNA integrates into the chromosome (2, 10, 13, 28). We have hypothesized that donor DNA is converted to a single-stranded form upon exiting from *H. influenzae* transformasomes, thus escaping restriction (2). Although less is known about the mechanism of *H. parainfluenzae* donor DNA processing, based on (i) the finding that donor DNA eludes restriction, (ii) conversion of one end of some donor molecules to a single-stranded form, and (iii) the presence of pore structure on the transformasomes, an analogous single-stranded integration mechanism is not unreasonable.

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